Gambierdiscus species diversity and community structure in St. Thomas, USVI and the Florida Keys, USA

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Highlights

- Species-specific FISH probes were used to assess community structure of *Gambierdiscus* species in field samples in the Caribbean.
- Multiple *Gambierdiscus* species shown to co-occur in natural populations, with low toxicity species dominating numerically.
- Shifts in *Gambierdiscus* community composition observed across geography and over seasons.

- Toxin-producing species *G. silvae* and *G. belizeanus* prevalent in St. Thomas but not in the Florida Keys.
- *Dictyota* may be a macrophyte vector for toxin transfer based on its widespread distribution, colonization by *G. silvae*, and palatability to at least some herbivore grazers.

Abstract

Ciguatera Poisoning (CP) is a widespread and complex poisoning syndrome caused by the consumption of fish or invertebrates contaminated with a suite of potent neurotoxins collectively known as ciguatoxins (CTXs), which are produced by certain benthic dinoflagellates species in the genera *Gambierdiscus* and *Fukuyoa*. Due to the complex nature of this HAB problem, along with a poor understanding of toxin production and entry in the coral reef food web, the development of monitoring, management, and forecasting approaches for CP has lagged behind those available for other HAB syndromes. Over the past two decades, renewed research on the taxonomy, physiology, and toxicology of CP-causing dinoflagellates has advanced our understanding of the species diversity that exists within these genera, including identification of several highly toxic species (so called "superbugs") that likely contribute disproportionately to ciguatoxins entering coral reef food webs. The recent development of approaches for molecular analysis of field samples now provide the means to investigate *in situ* community composition, enabling characterization of spatio-temporal species dynamics, linkages between toxic species abundance and toxin flux, and the risk of ciguatoxin prevalence in fish. In this study we used species-specific fluorescent *in situ* hybridization (FISH) probes to investigate *Gambierdiscus* species composition and dynamics in St. Thomas (USVI) and the Florida Keys (USA) over multiple years of sampling (2018-2020). Within each location, samples were collected

seasonally from several sites comprising varying depths, habitats, and algal substrates to characterize community structure over small spatial scales and across different host macrophytes. This approach enabled the quantitative determination of communities over spatiotemporal gradients, as well as the selective enumeration of species known to exhibit high toxicity, such as *Gambierdiscus silvae*. The investigation found differing community structure between St. Thomas and Florida Keys sites, driven in part by differences in the distribution of toxin-producing species *G. silvae* and *G. belizeanus,* which were present throughout sampling sites in St. Thomas but scarce or absent in the Florida Keys. This finding is significant given the high toxicity of *G. silvae*, and may help explain differences in fish toxicity and CP incidence between St. Thomas and Florida. Intrasite comparisons of *Gambierdiscus* distribution along a depth gradient found higher concentrations of *Gambierdiscus* spp. at deeper locations. Among the macrophytes sampled, *Dictyota* may be a likely vector for toxin transfer based on their widespread distribution, apparent colonization by *G. silvae*, and palatability to at least some herbivore grazers. Given its ubiquity throughout both study regions and sites, this taxon may also serve as a refuge, accumulating high concentrations of *Gambierdiscus* and other benthic dinoflagellates, which in turn can serve as source populations for highly palatable and ephemeral habitats nearby, such as turf algae. These studies further demonstrate the successful application of FISH probes in examining biogeographic structuring of *Gambierdiscus* communities, targeting individual significant toxin-producing species within the benthic assemblage, and characterizing species-level dynamics that are needed to describe and model ecological drivers of species abundance and toxicity.

Keywords: *Gambierdiscus*, ciguatera poisoning, ciguatoxin, Caribbean, dinoflagellate, benthic algae, harmful algal bloom

Abbreviations

CP, ciguatera poisoning; DAPI, 4′,6-diamidino-2-phenylindole; FISH, fluorescence *in situ* hybridization; LSU rDNA, large subunit ribosomal DNA; LSU rRNA, USVI, US Virgin Islands

1. Introduction

Ciguatera Poisoning (CP) is a widespread and complex poisoning syndrome caused by the consumption of fish or invertebrates from tropical and subtropical ecosystems that are contaminated with a suite of potent neurotoxins collectively known as ciguatoxins (CTXs). CTXs are produced by certain benthic dinoflagellate species in the genera *Gambierdiscus* and *Fukuyoa* (Chinain et al., 2021 and references therein), which inhabit the surfaces of a variety of macrophytes, as well as detritus on dead coral in tropical and subtropical coral reef ecosystems (Parsons et al., 2012; Cruz-Rivera and Villareal, 2006). Toxins originating in this benthic community enter the coral reef food web via grazing or filter feeding by herbivorous fishes and invertebrates, and are bioaccumulated and concentrated in coral reef food webs. Toxin levels are frequently highest in carnivorous reef fishes, many of which are targeted by commercial and recreational fisheries, although a wide variety of herbivorous species and several invertebrates have the potential to be ciguatoxic (Halstead, 1978; Laurent et al., 2012; Silva et al., 2015; Darius et al., 2018; Gatti et al., 2018).

Unlike many other HAB poisoning syndromes, CP outbreaks are not preceded or associated with large-scale planktonic blooms of a single causative species, but tend to be an ongoing problem in endemic regions in which a chronic "infratoxic" level of toxin can rapidly increase and cause outbreaks (Lee, 1980). Determining risk has been hindered by the variable nature of outbreaks, the cryptic diversity and varied toxin content of *Gambierdiscus* species potentially involved, and limited understanding of toxin production and entry in the coral reef food web. Consequently, the development of monitoring, management, and forecasting approaches for CP has lagged behind those available for other HAB syndromes, and prevention primarily relies on local ecological knowledge regarding fish species or fishing areas known to be risky (Chinain et al., 2010; André et al., 2021). Methods for rapid detection of CTXs in fish that could provide an affordable option for screening fish prior to consumption have been unsuccessful (thus far) due to the complex toxin chemistry and involvement of multiple toxins, as well as the lack of commercially available toxin standards and affordable and practicable methods for toxin detection. Field studies have been inconsistent in establishing linkages between genus-level cell abundance and toxicity (Liefer et al., 2021), or with environmental factors such as ocean warming (Chateau-Degat et al., 2005, Radke et al., 2013). Similarly, modeling efforts for *Gambierdiscus* have been limited to the genus level (Parsons et al., 2010) and are not yet able to forecast CP risk, which may be linked to one or more highly toxic species found within a cryptic assemblage comprising multiple low toxicity or non-toxic species.

Over the past two decades, however, renewed research on the taxonomy, physiology, and toxicology of *Gambierdiscus* and *Fukuyoa* has advanced our understanding of the diversity that exists within these genera, including identification of several highly toxic species that likely contribute disproportionately to ciguatoxins entering coral reef food webs. Currently the genera comprise 19 *Gambierdiscus* species and at least one ribotype, and four *Fukuyoa* species. Several of these species apparently are geographically widespread (e.g., *G. carpenteri*, *G. caribaeus*), while others appear to be more restricted in their distribution (Chinain et al., 2020; Chinain et al., 2021; Litaker et al., 2010). Early studies of ciguatera outbreaks in the Pacific showed that toxin production was limited to certain genetic strains of *Gambierdiscus,* leading to the suggestion that only these "super-producing" strains of *Gambierdiscus* were responsible for high ciguatoxin levels leading to outbreaks, i.e., the so-called "superbug hypothesis" (Holmes et al., 1991; Legrand, 1998). Over two decades later, this hypothesis has been substantiated with evidence of considerable toxicological diversity that has the potential to significantly influence the prevalence of ciguatoxins in food webs via shifts in community structure that favor growth of these particular superbug species or strains. Among them, *G. polynesiensis* is the most significant toxin-producer in the Pacific (Longo et al., 2019), while in the Caribbean Sea and Atlantic Ocean, *G. excentricus* and *G. silvae* are the most toxic species (Litaker et al., 2017; Pisapia et al., 2017; Robertson et al., 2018; A. Robertson, unpublished data), Recently, Mudge et al. (2023) identified and described a novel C-CTX analogue known as C-CTX5 that was produced by both *G. silvae* and *G. caribaeus* strains isolated from sites south of St. Thomas. This discovery represented the first report of a confirmed algal C-CTX precursor to the CTX congeners dominant in toxic fish that cause CP in the Caribbean region, with other studies limited to detecting bioactivity alone. Although a significant body of work has been generated on Pacific CTXs, there is much that is yet unknown about the toxicology of *Gambierdiscus* species in the Caribbean; however, the documentation of highly toxic *G. silvae* strains as well as identification of C-CTX5 production by *Gambierdiscus* species isolated from St. Thomas provides evidence of the source of toxins in fish that are responsible for CP in this region (Mudge et al., 2023; Robertson et al., 2018).

The recent development of approaches for molecular identification of *Gambierdiscus* species enable determination of species composition beyond genus-level estimation, and investigation of linkages between population dynamics and toxin dynamics. These include semi-quantitative qPCR assays for several *Gambierdiscus* species (Litaker et al., 2019; Nishimura et al., 2016; Kretzschmar et al., 2019; Vandersea et al., 2012), PCR-based restriction fragment length polymorphism (RFLP) typing methods for rapid differentiation of species or ribotypes (Lyu et al., 2017; Lozano-Duque et al., 2018), and high throughput sequencing to characterize benthic dinoflagellate community structure (D.L. Erdner, unpublished data; Smith et al., 2017). In addition to these approaches, a method for *Gambierdiscus* species identification and enumeration using fluorescence *in situ* hybridization (FISH) with species-specific probes was recently developed (Pitz et al., 2021). This method allows for accurate enumeration of high-toxicity species which may be present at low abundances, enabling integration of species abundance measurements into routine monitoring efforts as well as computational models of ciguatoxin fluxes in the food web.

In this study, we collected species-specific cellular abundance data using FISH probes from multiple sites within two ciguatera-endemic areas in the Caribbean: St. Thomas, US Virgin Islands (USVI), and the Florida Keys (USA). While both regions are impacted by ciguatera, the USVI has long been considered a hyperendemic region for CP, and the annual illness rates here are more than 20 times higher than in Florida (Radke et al., 2013, 2015). The eastern and southern regions in St. Thomas are considered particularly high risk by local fishers, who avoid harvesting fish from these areas (Loeffler et al., 2018).

This field investigation was part of a comprehensive and long-term effort to provide quantitative data on primary toxin-producing species and their distribution and dispersal pathways, identify environmental parameters (e.g., temperature, light, and wave action) as potential drivers of cell densities, and use these data to formulate a predictive model that relates species dynamics to ciguatoxin production and accumulation in seafood vectors. The goals of this study were to investigate community structure over spatiotemporal gradients, including species diversity and distribution of toxin-producing species within and between regions, and seasonal patterns in community structure. We sought to determine whether regional differences in *Gambierdiscu*s species composition could help explain the difference in reported CP incidence in St. Thomas versus Florida. Secondary goals included assessing community composition across different macrophyte hosts to explore potential host preferences. Moving forward, species-specific cellular abundance data will be integrated along with cellular toxicity values into models for ciguatoxin flux currently under development for both locations. The establishment of predictive capacity for forecasting CP events provided through these modeling efforts is an important and necessary next step in improving our ability to understand and manage the current and changing risks of CP.

2. Materials and Methods

2.1 Site descriptions

Field sampling was carried out seasonally from March 2018 to December 2020 (with some exceptions in 2020) at seven sites near St. Thomas, USVI and seven sites in the Florida Keys, USA (see Fig. 1, Table 1). All sites in St. Thomas were located south of the island, historically a source of toxic fish (Loeffler et al., 2018), and included nearshore and offshore locations. Black Point (BPT; 18.3445N, 64.98595W) is a nearshore fringing coral reef (7-16 m depth). Flat Cay (FLC; 18.31822N, 64.99104W) is a fringing coral reef on the leeward side of a small uninhabited island (11-16 m depth). Seahorse Reef (SHR; 18.29467N, 64.8675W) is a deep patch reef 2 km offshore of St. Thomas (19-22 m depth). The latter three sites are star coral (*Orbicella* spp.) reefs with diverse coral and sponge communities. Grammanik Tiger (GKT; 18.18885N, 64.95659W) is a primary bank mesophotic reef (37-41 m depth) with a rich assemblage of benthic and reef fish species. Hind Bank East (HBE; 18.20217N, 64.00158W) is a tertiary mesophotic bank (40-42 m depth) in the year-round no-take Marine Conservation District. South Capella (SCPS, SCPD; 18.26267N, 64.87237W) is an offshore site on a rise of the St. Thomas-St. John mid-shelf reef complex. To assess variability in the vertical distribution of *Gambierdiscus* communities, the South Capella location was sampled at two depths, permitting subsite comparisons (SCPS, 17-24m; SCPD, 35 m depth). Further site descriptions can be found in Ennis et al. (2019).

The Florida sites (Burrfish, Cheeca Rocks (Cheeca), Heine Grassbed (HGB), Keys Marine Lab (KML), Tennessee Reef Lighthouse (TRL), Research Only Area (TRL ROA), and deep (TRLD)) are all located in the vicinity of the middle Florida Keys, with all sites except Heine Grassbed and Keys Marine Lab located on the Atlantic side of the keys. Burrfish (24.99935N, 80.466733W) is a patch reef located in Hawk Channel on the Atlantic Ocean-side of the Florida Keys at an approximate water depth of 7 m. Macroalgae are common (particularly *Dictyota* spp.) followed by soft corals and sponges. Hard corals generally constitute <10% of the substrate. Cheeca Rocks (Cheeca; 24.904333N, 80.6157W) is another patch reef located just inshore of Hawk Channel on the Atlantic Ocean-side of the Florida Keys in water depths ranging from 1 m (top of coral heads) to 8 m (sandy bottom). Large, mounding corals dominate the substrate (>25% cover), including *Orbicella faveolata, O. annularis* and others. Soft corals and sponges are also common. Dominant macrophytes include *Dictyota* spp., *Halimeda* spp. and others (Brandt, 2016). Heine Grassbed (HGB; 24.859667N, 80.73816W) is a nearshore

Thalassia seagrass bed in Florida Bay (2 m depth). Siphonous chlorophytes are also present at HGB, including *Halimeda incrassata*, *Udotea* spp., and *Penicillis* spp. Keys Marine Lab (KML; 24.827N , 80.81405W) is a gulf-side hardbottom site, approximately 1-2 m deep. Dominant macrophytes include *Halimeda* spp., other siphonous chlorophytes, and *Sargassum* spp. Soft corals and sponges are common, as are *Thalassia testudinum* patches on top of sand/mud accumulations in depressions in the Pleistocene reef-matrix constituting the hard substrate. Tennessee Reef Lighthouse (TRL; 24.745N; 80.7812W) is a reef flat/crest site composed of soft corals, sponges, and macroalgae including turf algae, *Dictyota menstrualis*, and *Halimeda gracilis*. To assess variability in the vertical distribution of *Gambierdiscus* communities at a second location, TRL was sampled at three depths to permit subsite comparisons. The TRL Shallow and TRL ROA sites are at 7m and 6m, respectively, with the ROA site located within a designated no-take zone, and the TRL Deep is at ~14m**.**

2.2 Macroalgal Sample collection and Processing

Methods for macroalgal sample collection and processing are summarized in Parsons et al. (2017) and Pitz et al. (2021). The two or three most dominant macrophytes were sampled in triplicate at each site, at least 5 m apart from each other. Wet weights of algal grabs ranged from 5-233 g (avg 57.1 ± 41.6 g), and varied depending on algal availability, grab size, and algal type (calcified versus fleshy). Algal taxa collected at the Florida Keys sites included *Dictyota cervicornis* (Kützing 1859), *D. menstrualis* ((Hoyt) Schnetter, Hörning & Weber-Peukert 1987), *Halimeda gracilis* (Harvey ex J. Agardh 1887), *H. incrassata* ((J. Ellis) J.V. Lamouroux 1816), *Laurencia gemmifera* (Harvey 1853), *L. intricata* (J.V. Lamouroux 1813), S*argassum* spp. (C. Agardh, 1820), and the seagrass, *Thalassia testudinum* (K.D. Koenig 1805). In St. Thomas, algal taxa collected included *D. menstrualis*, *Halimeda spp.*, *Lobophora* spp. (J. Agardh, 1894), and

Sargassum spp. Macrophyte samples were shaken and filtered through 200 and 20 µm sieves (PVC; Nitex mesh; 6.3 cm diameter), refilled with 20 µm-filtered ambient seawater, and shaken and filtered an additional four times. Epiphytic material collected on the 20 µm sieve was backwashed into a 15 mL centrifuge tube using ambient filtered seawater and brought to a volume of 14 mL. Each sample was preserved with 750 µL of formalin and stored for at least five minutes, but no more than 12 hours. The sample was then centrifuged at 3000 *g* for 10 minutes, the supernatant removed via aspiration, and each sample was then re-suspended in ice-cold methanol (volume was adjusted to accommodate sample density). Samples were stored at -20°C for at least 24 hours prior to whole cell hybridization (Section 2.3). Macrophyte samples were blotted dry and weighed (g wet weight) on a Mettler Toledo AL204 or similar balance and then identified, using keys as necessary (e.g., Littler & Littler, 2000). Over the course of the study, a total of 480 samples were analyzed, 220 from the Florida Keys and 260 from St. Thomas (see Table 2 for details).

Benthic cover data were collected at sites in St. Thomas, and at the Tennessee Reef Lighthouse (shallow and deep) and Burrfish sites in the Florida Keys. Each study site was estimated using digital video along six randomly sited permanent transects as described in Smith et al. (2013). Each St. Thomas transect was 10 m in length and marked with steel rods (25 m for Florida Keys sites), with transects spaced at least 3 m apart (5 m for Florida Keys sites). Digital video was recorded perpendicular to the substrate and resultant images were cut into non-overlapping images, typically 15 per transect. Fifteen random points were placed on the image using Coral Point Count software (Kohler & Gill, 2006) and characterized to the lowest identifiable taxonomic or abiotic level by a trained expert. Cover of each category (i.e., Coral, Gorgonians, Sponges, Zoanthids, Macroalgae, Coralline Algae, Dead Coral with Turf Algae, Non-living Substrate, Other Living) was calculated for each transect by dividing the number of occurrences by the total number of points surveyed. Macroalgal cover was partitioned into % Crustose Coralline algae, % *Dictyota* spp., % *Lobophora variegata*, % *Halimeda* spp., % *Sargassum* spp. and % other algae (which included Turf algae).

2.3 Fluorescent *in situ* hybridization and cell enumeration

Benthic samples were analyzed using fluorescent probes designed to target the large subunit (LSU) region of rRNA of the five known Atlantic species of *Gambierdiscus: G. belizeanus, G. caribaeus*, *G. carolinianus, G. carpenteri* and *G. silvae* (Pitz et al., 2021). This FISH method uses probe multiplexing with multiple fluors, which were selected based on their labeling effectiveness and their compatibility due to emitting light in distinct spectral regions. This approach allows for the differentiation and enumeration of multiple species within a single sample, and provides community characterization while preserving the quantitative strength of microscopic enumeration.

The five species monitored in this study were selected based on prior extensive characterization of the *Gambierdiscus* communities found at these sampling sites, which included species identification of > 900 isolates over multiple years using DNA sequencing and RFLP typing (Lyu et al., 2017; Lozano-Duque et al., 2018) that were established in culture for microsatellite studies on *G. caribaeus* (Sassenhagen et al., 2018), as well as high throughput sequencing of samples collected monthly over a two-year period to characterize the broader benthic community (D.L. Erdner, unpublished data).

FISH hybridization processing followed methods outlined in Pitz et al. (2021), and are summarized below. Samples were centrifuged (5 min x 10000 *g*) to pellet contents and overlying methanol was aspirated, taking care to leave the pellet undisturbed. The pellet was resuspended in 1 mL hybridization buffer (5× SET, 0.1% IGEPAL CA-630, Poly A 10 mg mL^{-1}, 10% formamide) and incubated at room temperature for five minutes. Probe was added at a final concentration of 1.6 ng μL^{-1}, and samples were incubated in the dark on a heating block at 53 or 55°C (Tm-dependent) for one hour, with motion set at 350 rpm. Following the incubation, samples were pelleted via centrifugation, and hybridization buffer was aspirated and replaced with 1 mL wash buffer (0.2× SET solution). Samples were incubated at room temperature for five minutes and centrifuged. Wash buffer was aspirated and pellet was resuspended in 1 mL of 5× SET solution containing 10 µL of a working stock solution of Calcofluor White (10 mg mL^{-1}). The use of Calcofluor White to stain cells enables the initial identification of *Gambierdiscus* at the genus level when field samples are observed under the DAPI filter set. This modified centrifugation method has proven effective for processing large numbers of samples, and quality of labeling is comparable to the manifold filtration method commonly used for FISH analysis of other HAB species (e.g., *Alexandrium* sp.; Anderson et al., 2005).

Following hybridization, each sample was loaded into a Sedgewick-Rafter slide in 1 mL aliquots for subsequent identification and enumeration under fluorescence microscopy. All samples in this study were analyzed using a Zeiss Axio Vert.A1 inverted microscope (Carl Zeiss AG, Oberkochen, Germany) at 100×. Initially, all slides were scanned using the DAPI filter set. When a *Gambierdiscus* cell was detected at the genus level, the Cy3, Texas Red, and FITC filters were applied in sequence to determine whether the cell had been labeled with a fluorophore. Fluorescently labeled species were distinguished by fluor brightness, intensity, and color when observed under the three filter sets (See Supplementary Fig. 1). For example, cells labeled with Cy3 (or Alexa Fluor® 532) displayed a bright, orange appearance when viewed through the Cy3 filter set. In contrast, they appeared less visible and darker under the FITC and Texas Red filters. Cells labeled with the Texas Red fluorophore (or Alexa Fluor® 594) exhibited a vibrant red hue under the Texas Red filter but appeared as a faded red under the Cy3 filter and were barely visible under the FITC filter. Cells labeled with FITC (or Alexa Fluor® 488) appeared as a bright, chalky green under the FITC filter but were barely visible under the other two filters. Unlabeled cells were faintly visible under all three filter sets. To ensure the effectiveness of the multiplexing approach, each cell was examined under all three filter sets, and labeling was differentiated by color intensity and brightness. Subsequently, the filter setting was returned to DAPI, and scanning resumed until the next *Gambierdiscus* cell was located.

2.4 Statistical Analyses

Gambierdiscus species community composition across sites and sampling time points were explored in a variety of data subsets using cluster analyses and non-metric multidimensional scaling. These data were subset by year to explore variation within each sampling year; general location (Florida, St. Thomas); macroalgal substrate; and even site depth to explore all potential relationships. Multivariate similarities were measured with an ANOSIM test, although all multivariate results were not significant. These tests were performed using PRIMER 7 version 7.0.21 (Clarke & Gorley, 2015). All reports of cells per gram were calculated by averaging the cells per gram of both Group 1 (*G. caribaeus*, *G. belizeanus*) and Group 2 (*G. carpenteri*, *G. carolinianus*, *G. silvae*/*G. polynesiensis*) multiplex counts (see Pitz et al., 2021) and then averaging across selected categories (date, year, site, subsite, algae species). Comparisons of *Gambierdiscus* abundance between shallow and deep sites were performed using R version 4.1.1

and RStudio 2021.09.0+351 "Ghost Orchid" Release. Given that macroalgal taxa distribution was patchy over time, abundance data collected from all algal substrates were compiled and analyzed rather than analyzing abundances from each host separately. Data were first tested for normality using the Shapiro-Wilk test and for homogeneity of variance using Q-Q plots of residuals. A square root transformation was used when these data did not meet the assumptions. Values above Q3 + 3xIQR or below Q1 - 3xIQR were considered to be extreme outliers and removed before analyses (Q1 and Q3 are the first and third quartile, and IQR = Q3 - Q1 the interquartile range). One-way ANOVAs were used to compare overall differences in the average cells per gram of wet weight algal substrate between the shallow and deep sites followed by Tukey's post hoc test for multiple comparisons. The TRL and SCP shallow versus deep sites were analyzed separately as the depths at each site were not directly comparable. Two-way ANOVA was used to explore *Gambierdiscus* abundance among sites and algal taxa, although the data for at least one combination of site and algal taxa for both TRL and SCP were not normally distributed even after applying multiple transformations. Results were considered to be significant at the $a = 0.05$ level.

3. Results

3.1 Macroalgal community composition

In St. Thomas, *Lobophora* (n=172) was the most common macrophyte host throughout sites and over seasons, and thus was collected most frequently, followed by *Dictyota* (n=124) and *Sargassum* (n=23). In general, the algal communities at nearshore sites shallower than 20 m were dominated by macroalgae in the genus *Dictyota* and turf algae, whereas deeper, offshore sites were dominated by *Lobophora* spp. and turf algae. Hard corals generally constituted between ~12-30% of the substrate. In the Florida Keys, *Halimeda* (n= 87) and *Dictyota* (n=79) were most frequently encountered and collected, and macroalgal communities were generally more diverse and variable across sampling sites than observed in St. Thomas. One site in the Florida Keys, HGB, was dominated by *Thalassia testudinum*, and was the only location where seagrass was collected. *Laurencia* was present and collected (n=6) at the shallower sites including Cheeca and KML. *Halimeda incrassata* tended to be more abundant in nearshore and bayside sites (Cheeca, KML, HGB) whereas *Halimeda opuntia* was more common at the deeper, offshore sites (TRL, TRL ROA, and TRL Deep).

Seasonality was more pronounced among macroalgae in the Florida Keys versus St. Thomas. For example, *Laurencia* tended to be most abundant at the Keys sites in the summer and fall months (July-November), and *Thalassia testudinum* was more abundant at HGB in the summer months (June-July). Algal abundance was more stable at the offshore (TRL) sites versus the nearshore and bayside sites in the Keys (KML, HGB and Cheeca). In both regions, *Dictyota* displayed seasonal cycles of abundance, being most abundant in winter months (December-February) and rare/absent in summer (~June-September).

3.2 *Gambierdiscus* spp. abundance and regional distribution

Gambierdiscus spp. were detected in all but 26 of the 480 samples collected in this study. Abundances were highly variable over time, within and among sites, and across macrophyte hosts (Table 3; Supplementary Fig. 2). Overall *Gambierdiscus* abundance ranged from 0 - 351 cells g wet weight^{-1} , with highest cell concentrations observed at the Florida Keys sites. In St. Thomas, highest cell concentrations of *Gambierdiscus* spp. (22.7 ± 12.8 cells g *Dictyota* $^{-1}$) were found in December 2020 at BPT (Supp. Fig. 1). In Florida, the highest mean cell concentration of *Gambierdiscus* spp. (144.6 ± 21.3 cells g *Thalassia* $^{-1}$) was found at HGB in October 2020 (data not shown). Across all sites in both regions, either *G. caribaeus* or *G. carolinianus* were most abundant, generally comprising 80% or more of cells enumerated (Figs. 2 & 3, Table 3). In St. Thomas, highest proportional abundance of *G. caribaeus* was 86.6%, found on *Dictyota* samples collected from SCPS (November 2019), while highest proportional abundance of *G. carolinianus* was 91.6%, on *Lobophora* samples from SCPS (December 2020). These two species dominated *Gambierdiscus* populations at many of the Florida Keys sites as well as during all time periods (Fig. 3). There, maximum proportional abundance of *G. caribaeus* was 88.8%, found on *Dictyota* from TRL Deep (October 2018), while *G. carolinianus* comprised 100% of *Gambierdiscus* cells enumerated in *Sargassum* samples collected from TRL ROA (October 2020). At the grass bed sampling site in Florida (HGB), these two species comprised over 99.4% of the cells enumerated (Fig. 2, Table 3). *Gambierdiscus carpenteri* was found in very low proportions across all sampling sites in both regions, generally comprising <3.4% of cells enumerated. Maximum proportional abundance of this species was 58.3% found on *Dictyota* collected from TRL ROA (June 2018).

Gambierdiscus silvae and *G. belizeanus* were absent or scarce at most sites in the Florida Keys (Figs. 2 & 3). Abundances of these two species rarely exceeded 8% of species enumerated in a given sample, and these species were also entirely absent from some of the sampling sites (e.g., Cheeca, KML, HGB; Table 3). When present, *G. silvae* was most frequently found in samples collected from the offshore Tennessee Reef Lighthouse sampling locations (Fig. 2, Table 3), and maximum proportional abundance of this species was 23.8% found on *Dictyota* samples collected from TRL Shallow (June 2019). In contrast with the Florida Keys sites, *G. silvae* and *G. belizeanus* were present at all sampling sites in St. Thomas and at higher proportions. Maximum proportional abundance of *G. silvae* was 65.7% at SHR (March 2019, *Dictyota*), while *G. belizeanus* was most abundant (30%) in samples collected from FLC (September 2018, *Lobophora*). An exploratory analysis using non-metric multidimensional scaling showed community structuring between sites in St. Thomas and Florida, driven in part by the prevalence and higher proportional abundance of *G. silvae* and *G. belizeanus* in St. Thomas (Fig. 4).

3.3 *Gambierdiscus* spp. distribution and host macrophytes

Benthic communities at most of the sampling sites were dominated by macroalgae. At sites in St. Thomas, macroalgae comprised between 58 - 70% of benthic cover, fluctuating over time. In the Florida Keys, macroalgae comprised between 20 - 45% of benthic cover at all sites with the exception of HGB, which was dominated (up to 50%) by seagrass (*Thalassia* spp.; data not shown). *Dictyota*, *Halimeda*, and *Lobophora* were the most frequently collected macrophyte taxa, based on their widespread occurrence across the sampling sites (Fig. 5). *Dictyota* was found at five of St. Thomas sites, including both SCP subsites, and five Florida Keys sites, including all three TRL subsites. *Lobophora* was widespread across all sites in St. Thomas, with the exception of BPT, but was absent from the Florida Keys sites. *Halimeda* was widespread in the Florida Keys, and was collected at all sampling sites, but in St. Thomas was only collected at BPT. *Sargassum* was only collected at SHR and TRL in St. Thomas and the Florida Keys, respectively, and was largely rare or absent from other sites. *Laurencia* (red algae) and *Thalassia* (seagrass) were both absent in St. Thomas, and were only collected in the Florida Keys. Both macrophytes were found at KML, while *Thalassia* was only found at HGB, and *Laurencia* was only found at Cheeca. No statistically significant patterns in the distribution of *Gambierdiscus* spp. across host macrophytes were observed. In St. Thomas, *G. silvae* was found on all macrophyte taxa collected, with the exception of single samples of *Halimeda* and *Lobophora* collected from BPT (Table 3, Fig. 5). In both St. Thomas and the Florida Keys, maximum proportional abundance of *G. silvae* was on *Dictyota*, while *G. belizeanus* was most abundant on *Lobophora* and *Dictyota* collected from St. Thomas and Florida, respectively. In St. Thomas, *G. silvae* was found on nearly all macrophyte taxa collected (Fig. 5), with maximum proportional abundances (66%) on *Dictyota* collected from SHR in March 2019, where it was the dominant species present. Proportional abundance of *G. belizeanus* was highest (30%) on *Lobophora* collected from FLC. In the Florida Keys, highest proportional abundance of both *G. silvae* (23.8%) and *G. belizeanus* (17.8%) was found on *Dictyota* collected from TRL Shallow.

3.4 Shallow and deep subsite comparisons

At the two sites with shallow and deep comparisons (Tennessee Reef Lighthouse, TRL in Florida; South Capella, SCP in St. Thomas), there were significantly higher abundances of overall *Gambierdiscus* spp. at the deep subsites in both locations across all algal taxa used in these comparisons. At TRL, there was a significant impact of subsite on *Gambierdiscus* abundance (One-way ANOVA, F= 7.07, p =0.001), and cell concentrations at the two shallow sites (TRL Shallow, 8m; TRL ROA, 6 m; see Fig. 6 and Table 2) were significantly lower than at the deep sites (TRL Deep, 14 m; TukeyHSD, p= <0.05). *Gambierdiscus* abundance was not significantly different between the two shallow sites (TRL Shallow and TRL ROA), indicating that there was no apparent impact of the protected area on cell densities (TukeyHSD, p= 0.521). Highest overall cell concentrations on *Dictyota* and *Halimeda* at these subsites were found in samples collected at TRL Deep (October 2018) and TRL ROA (October 2018), respectively. Statistical comparisons of *G. silvae* abundance was not possible due to low detection resulting in dramatically zero-inflated data, but this species was more frequently detected in samples from TRL Deep (19%), followed by TRL Shallow (17%), and TRL ROA (5%). A similar trend was observed at South Capella in St. Thomas, where shallow (SCPS, 21m) and deep (SCPD, 35m) sub-sites were also sampled. Here, *Gambierdiscus* spp. cell concentrations were significantly higher at the deep sites compared to shallow sites (One-way ANOVA, F=5.522, p=0.0214).

In St. Thomas, *G. silvae* was prevalent at both subsites and on both macrophytes, and occurred in 41.9% of samples collected from the shallow subsite (SCPS), and 27.8% of samples collected at depth (SCPD). Highest abundances of this species on *Dictyot*a and *Lobophora* were found in samples collected at SCPD (July 2019) and SCPS (March 2019), respectively.

Discussion

Recent advances in the taxonomy and toxicity of *Gambierdiscus* spp. and development of molecular approaches to species detection in field samples provide a path forward to addressing long-standing questions in ciguatera research regarding the link between the *Gambierdiscus* assemblages at a particular site and toxins entering the food web. In particular, the identification of highly toxic "superbug" species along with a technique for quantitative detection provides a critical missing component in monitoring approaches necessary for developing predictive capabilities for CP risk. Species level quantification of *Gambierdiscus* communities also provides a powerful new tool for characterizing species distribution at local and regional scales, investigating linkages between the dynamics of toxin-producing species and CP prevalence, and identifying ecological drivers and timing of toxin flux. This investigation used newly developed molecular probes to explore *Gambierdiscus* species diversity and community structure over spatio-temporal gradients in the Caribbean, including across multiple macrophyte host taxa.

We used fluorescence *in situ* hybridization (FISH) to quantify abundance of Caribbean *Gambierdiscus* species at multiple sampling sites at two locations: the Florida Keys, USA, and St. Thomas, USVI. Although ciguatera affects both areas, the USVI have traditionally been classified as a hyperendemic region for CP, with annual illness rates exceeding those in Florida by over 20 times (Radke et al., 2013, 2015). CP in southern Florida has an estimated annual incidence of 28-84 per 100,000 (Miami-Dade and Monroe Counties), with the majority of cases caused by fish caught in the Bahamas and Florida Keys (Radke et al., 2015). CP incidence in St. Thomas is estimated to be considerably higher, up to 1,200 per 100,000 (Radke et al., 2013) cases annually. The eastern and southern regions in St. Thomas are considered particularly high risk by local fishers, who avoid harvesting fish from these areas due to local knowledge of the most ciguatoxic zones around the island. Studies of ciguatoxin prevalence in commonly marketed and consumed species from around the island concurred with spatial patterns in CP risk identified by fishers, confirming ciguatoxic zones south, east, and west of the island (Loeffler et al., 2018). In this study we explored whether regional differences in *Gambierdiscu*s species composition could help explain this regional difference in CP incidence.

Five *Gambierdiscus* species were selected for enumeration in samples collected seasonally from March 2018 to December 2020, based on their predominance at both sampling locations determined by culture-based identification efforts (Lozano-Duque et al., 2018, Lyu et al., 2017; Pitz et al., 2021; Richlen, Erdner, unpublished data). Past efforts included characterization of >900 isolates from St. Thomas and the Florida Keys over multiple years of sampling using DNA sequencing and RFLP typing (Lozano-Duque et al., 2018, Lyu et al. 2017). Additional species detected at low proportions at the study sites through culture-based identification efforts include *Gambierdiscus* sp. ribotype 2, which was identified in both St.

Thomas (five isolates) and the USVI (one isolate), and *Fukuyoa yasumotoi* (one isolate) which was identified in the Florida Keys. Neither *G. scabrosus* or *G. excentricus* have ever been observed in our culture-based analyses, nor in high throughput sequencing analyses currently underway to characterize the broader benthic community (D.L. Erdner, unpublished data).

The utilization of FISH with species-specific molecular probes offers direct determination of community composition and abundance in field samples while preserving the accuracy of microscopic cell counts (Supplementary Fig. 1; Pitz et al., 2021). A total of 480 field samples collected from 2018-2020 were analyzed (260 from seven sites in St. Thomas, and 220 from seven sites in Florida; see Table 1), representing a significant expansion of previous investigations of *Gambierdiscus* community structure, and to our knowledge, comprises one of the largest species-level datasets of *Gambierdiscus* abundance in the world. In addition to coverage across large spatial gradients, we investigated community differences between deep and shallow water habitats at two sampling locations (SCP, St. Thomas; TRL, Florida) and across multiple macroalgal taxa to explore the distribution of *Gambierdiscus* species across different substrates. These studies are part of a comprehensive and long-term effort to provide quantitative data on primary toxin-producing species and their distribution for numerical models relating species dynamics to ciguatoxin uptake and accumulation in seafood vectors.

During all three years of sampling, *G. caribaeus* and *G. carolinianus* were proportionally most abundant across all sampling sites and during all time periods in St. Thomas and the Florida Keys, and together these two species often comprised 90% or more of cells enumerated. These findings are similar to results of culture-based assessments of *Gambierdiscus* communities carried out previously (Lozano-Duque et al., 2018, Lyu et al., 2017), in which these two species were most frequently isolated from field samples collected from multiple locations in the Florida

Keys and St. Thomas over a period of several years. These two species have been found in high proportions in *Gambierdiscus* communities sampled elsewhere in the Caribbean, including along the south-central coast of Cuba, where *G. caribaeus* and *G. carolinianus* (along with *G. belizeanu*s) were the predominant species (Díaz-Asencio et al., 2019). This latter assessment was carried out using PCR analysis of samples collected from three sites comprising multiple depths as well as multiple macrophyte hosts. These species also have an apparent wide geographic distribution in the Caribbean and beyond - both have been reported from multiple locations throughout the Caribbean Sea, as well as the Gulf of Mexico and Atlantic Ocean (reviewed by Chinain et al., 2020). *Gambierdiscus caribaeus* appears to have the broadest distribution globally, and has also been reported from multiple locations throughout the Pacific, as well as several coastal locations in Asia (Chinain et al., 2020). Current data has not identified G. *carolinianus* as a significant toxin-producer (Litaker et al. 2017; Pisapia et al., 2017), but *G. caribaeus* (along with *G. silvae*) from St. Thomas was recently identified as producing C-CTX5, a novel C-CTX analogue (Mudge et al., 2023).

Despite these similarities, comparison of community composition between the Florida Keys and St. Thomas found several important differences between these regions. One notable difference was the higher prevalence of *G. belizeanus* and the highly toxic species *G. silvae* in samples collected from St. Thomas, particularly in samples collected in March 2019 (Fig. 2, 3, Table 3). In Florida these two species were present at extremely low abundances, and these species were also entirely absent from some of the sampling sites (e.g., Cheeca, KML, HGB; Table 3). In contrast, these species were found at every location sampled in St. Thomas, and in higher proportional abundance compared to locations in the Florida Keys. Notably, the toxin quotas of "superbug" species such as *G. silvae, G. excentricus,* and *G. polynesiensis* can be over

1000-fold higher than lower toxicity species such as *G. carolinianus* and *G. caribaeus* (Chinain et al., 2010; Litaker et al., 2017; Robertson et al., 2018). These findings verify that *G. silvae* is likely an important contributor to the *in situ* benthic toxicity measured previously in St. Thomas by Liefer et al. (2021), as well as the prevalence of toxins in fish from locations southwest of the island (Loeffler et al., 2018). The prevalence of *G. silvae* in St. Thomas may also help explain the marked differences in fish toxicity and CP incidence between Florida and St. Thomas, while changes in proportional abundance over small spatial scales would determine variability within regions. However, we cannot rule out that an as yet undocumented or undiscovered highly toxic species may also contribute to these spatial differences.

To assess variability in the vertical distribution of *Gambierdiscus* communities, SCP (St. Thomas) and TRL (Florida) were sampled at two depths, permitting subsite comparisons in both regions. SCP, an offshore site on the St. Thomas-St. John mid shelf reef complex, was sampled at depths of 16 m (SCPS) and 35 m (SCPD). In Florida, three subsites were sampled at Tennessee Reef Lighthouse, a reef flat/crest site in the vicinity of Long Key. Two shallow subsites were at ~6-8 m depth, one of which was located within a no-take, research only area (TRL ROA), while the deeper site was at 15 m; deeper than the two shallow subsites in Florida, but not as deep as either of the SCP sites. We hypothesized that deeper sites may harbor higher concentrations of *G. silvae* based on prior studies documenting higher concentrations of benthic dinoflagellates, including *Gambierdiscus*, at depth, as well as physiological studies indicating a lower thermal tolerance of this species compared to other *Gambierdiscus* species tested (Richlen & Lobel, 2011; Xu et al., 2016). In both St. Thomas and Florida, maximum monthly mean temperatures decrease with depth (Leichter et al., 1996, Smith et al., 2016) while warm-season seawater temperatures in shallow water at the sampling sites in both regions routinely exceed

28°C, which in laboratory experiments was associated with steep declines in growth rates of *G. silvae*. In contrast, *G. caribaeus* and *G. carolinianus* generally exhibited higher temperature optima for growth as well as a wider range of thermal tolerance, with some strains capable of sustaining growth at temperatures above 32°C (Kibler et al., 2012; Xu et al., 2016). In both St. Thomas and Florida, overall *Gambierdiscus* spp. cell concentrations were higher at the deeper subsites, compared to shallow subsites suggesting that these deeper locations may host higher concentrations of toxic dinoflagellates and produce fish with higher toxicity. In Florida, overall *Gambierdiscus* abundance was not significantly different between the two shallow sites (TRL Shallow and the no-take site TRL ROA), indicating that there was no apparent impact of the protected area on cell concentrations.

In both regions, multiple algal taxa were collected where possible to examine the diversity and distribution of *Gambierdiscus* communities over different substrates, and to explore the epiphytic associations between *Gambierdiscus* species and the host macroalgae found in each region. In particular, we sought to determine if certain macrophytes might serve as primary ciguatoxin vectors, based on their biomass, palatability to grazers, and colonization by toxic *Gambierdiscus* species (*G. silvae* and *G. belizeanus*). Prior field studies comparing *Gambierdiscus* spp. abundance across multiple macrophyte hosts and artificial substrates found that genus level cell concentrations on macrophytes were variable, with some indication of substrate preference; however, epiphyte-host preferences were location specific and not consistent across sites (Parsons et al., 2017). These results may reflect the presence of multiple *Gambierdiscus* species and differences in the way particular *Gambierdiscus* species associate with different macrophyte hosts (Rains & Parsons, 2015), which would interact with other host and site specific factors in determining the distribution of *Gambierdiscus* species across varying spatial scales.

Over the three years of field sampling, *Dictyota*, *Halimeda*, and *Lobophora* were the most frequently collected macrophyte taxa due to their occurrence across sampling sites; of these, *Dictyota* was the most widespread. In St. Thomas, *G. silvae* was found on nearly all macrophyte taxa collected, with maximum proportional abundances found on *Dictyota*, while *G. belizeanus* was most abundant on *Lobophora.* In Florida*,* highest proportional abundances of both *G. silvae* and *G. belizeanus* were found on *Dictyota*. Based on these field observations, *Dictyota* (and to a lesser degree, *Lobophora*) may be a key vector for toxin transfer, given its high biomass and cover, widespread spatiotemporal distribution across both sites and regions, and colonization by two prominent toxin-producing species - *G. silvae* and *G. belizeanus.* This is supported by earlier laboratory and field studies, which identified *Dictyota* as an important algal taxon in ciguatoxin uptake by at least some herbivore grazers, as well as "browsers" that may selectively feed on algal epiphytes (Burkpile & Hay, 2009; Cruz-Rivera & Villareal, 2006; Duran et al., 2019). Laboratory experiments examining host preferences (*Gambierdiscus* behavior and attachment) in the context of host palatability identified *G. belizeanus* - *Dictyota* as an important species-host pairing based on the epiphytic behavior (growth and attachment) of *Gambierdiscus* cells towards this host (Rains and Parsons, 2015). *Gambierdiscus silvae* was not included in these experiments and at that time little was known regarding the toxin-producing capabilities of this species. An earlier analysis of marine algal–herbivore interactions by Cruz-Rivera and Villareal (2006) reviewed distribution patterns of *Gambierdiscus* on algal hosts, also identifying *Dictyota* as harboring extremely high cell concentrations of *Gambierdiscus* spp. Such macroalgal taxa might ultimately act as toxin sources or sinks, depending on their chemical and structural (e.g., calcification) defenses that deter grazing by herbivores. While *Dictyota* is chemically defended and not as palatable to grazers as turf algae, this taxa is still consumed by some herbivores (Cruz-Rivera and Villareal, 2006). Based on these prior studies as well as our field observations, we also concluded that *Dictyota* may be a likely vector for toxin transfer based on its high biomass and cover, widespread spatiotemporal distribution across both sites and regions, ready colonization by *G. silvae* and *G. belizeanus*, as well as palatability to at least some grazer communities. Given its ubiquity throughout both study regions and sites, this taxon may also serve as a refuge, accumulating high concentrations of *Gambierdiscus* and other benthic dinoflagellates could serve as source populations for colonizing nearby palatable and ephemeral habitats such as turf algae.

Conclusions

Unlike many other HAB poisoning syndromes, CP is not associated with large scale blooms of a single causative organism, but is often a chronic issue linked to the presence and dynamics of one or more toxic species within a cryptic assemblage. The recent development of approaches for molecular identification of *Gambierdiscus*, such as FISH enumeration utilized here, provides the means to determine *in situ* species composition of *Gambierdiscus* communities over varying spatial and temporal scales, and could also be applied to investigate linkages between toxic species abundance and toxin flux, and characterize environmental drivers of species dynamics. Our findings show differences in *Gambierdiscus* communities between St. Thomas (USVI) and Florida Keys (USA) sites that were driven in part by higher proportional abundance of toxin-producing species *G. silvae* and *G. belizeanus* in the USVI compared to the Florida Keys. This finding is significant given the high toxicity of *G. silvae*, and may help explain differences in fish toxicity and CP incidence between St. Thomas and Florida. This substantial dataset also provides species-level dynamics for modeling efforts currently underway to describe and predict species abundance and toxin dynamics, and establishes a feasible approach for targeting individual toxic “superbug” species that are often found at low abundance within benthic assemblages.

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Table Captions

Table 1. Location and depth of study sites in the Florida Keys, USA and St. Thomas (USVI) sampled from 2018-2020.

Table 2. Number of samples analyzed for *Gambierdiscus* species abundance from study sites in the Florida Keys and St. Thomas (USVI) from 2018-2020.

Table 3. Average *Gambierdiscus* species abundance in samples collected from multiple macroalgal hosts and across multiple sampling sites in the Florida Keys and St. Thomas.

Figure Captions

Figure 1. Map of sampling sites in the Florida Keys, USA (top panel) and St. Thomas, USVI (bottom panel).

Figure 2. Proportional abundance of *Gambierdiscus* species at sampling sites in the Florida Keys, USA (top panel) and St. Thomas, USVI (bottom panel) from 2018-2020.

Figure 3. Temporal trends in *Gambierdiscus* community composition in the Florida Keys (USA) and St. Thomas (USVI) from 2018-2020.

Figure 4. NMDS plot of *Gambierdiscus* species from samples collected in 2018-2020, with

species vectors displayed. Green circles and blue triangles represent sites from the Florida Keys (USA) and St. Thomas (USVI), respectively.

Figure 5. *Gambierdiscus* species distribution across algal taxa in St. Thomas (USVI) and the Florida Keys (USA).

Figure 6. *Gambierdiscus* species abundance and community composition on dominant algal taxa collected from shallow versus deep sites in St. Thomas (USVI) and the Florida Keys (USA). Box and whisker plots depict the overall *Gambierdiscus* abundance including unlabeled cells found on each algal species. Boxes are the interquartile range, the middle line is the median value and whiskers are the variability outside the first and third quartile. Donut charts depict the overall proportional abundance of each *Gambierdiscus* species identified.

Supplementary Figure 1. Demonstration of labeled *Gambierdiscus* cells hybridized with species-specific probes using multiplexed fluorophores. Each row displays a single *Gambierdiscus* cell under the DAPI filter, used for genus-level identification, followed by Cy3, Texas Red and FITC filters to view labeling by species-specific fluorophores. The species identification and fluorophore labeling of each cell are indicated on the left-hand side of the figure. Group labels indicated probe multiplexing combinations used in the hybridizations (see Pitz et al., 2021).

Supplementary Figure 2. Time series of *Gambierdiscus* spp. abundance on *Dictyota* macroalgae collected from the Florida Keys (top panel) and St. Thomas, USVI (bottom panel).

Boxes are the interquartile range, the middle line is the median value and whiskers are the variability outside the first and third quartile.