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Effects of grazing, nutrients, and depth on the ciguatera-causing dinoflagellate *Gambierdiscus* in the US Virgin Islands

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ABSTRACT: Ciguatera fish poisoning in humans is a serious and widespread syndrome associated with the consumption of reef fishes that have accumulated lipid-soluble toxins known as ciguatoxins. These toxins are piscine metabolites of ciguatoxin precursors produced by benthic dinoflagellates in the genus *Gambierdiscus*. This investigation employed a novel experimental approach to identify and characterize the environmental factors and their interactions that influence the dynamic balance between cellular growth and loss of *Gambierdiscus* populations *in situ*. Field studies were conducted in St. Thomas (US Virgin Islands) at 3 sites and 2 depths (10 and 20 m). At each site and depth, *Gambierdiscus* was subjected to treatments designed to reduce grazing pressure (disturbance and removal) and elevate nutrient availability to elicit a population abundance response attributable to one of these treatments. We hypothesized that *Gambierdiscus* abundance would respond positively to increased nutrient availability, increasing depth (reduced water motion), and decreased grazing pressures. We found communities of *Gambierdiscus* were significantly higher by, on average, 138% when the effects of grazing were limited ($p = 0.0002$). Among sites, the effects of depth and nutrients on *Gambierdiscus* populations were not significant. The significant effect of grazing and disturbance observed in this study suggests that changes in reef herbivore and detritivore feeding selectivity and grazing rates may have large impacts on the areal density of *Gambierdiscus* in natural systems. Whether or not reduced grazing rates or disturbances translate into higher cell (toxin) ingestion rates for consumers and ultimately cause changes in toxicity for humans is unknown and in need of further investigation.

KEY WORDS: Ciguatera fish poisoning · *Gambierdiscus* · Caging · Grazing · St. Thomas · Coral reefs · Fish survey · Management

INTRODUCTION

Ciguatera fish poisoning (CFP) is a circumtropically distributed syndrome caused by the ingestion of ciguatoxins. These toxins are most commonly encountered when humans consume fish associated with shallow tropical marine environments where these toxins are produced. The ciguatoxins found in CFP-implicated fish arise from many different precursor ciguatoxins (e.g. C-CTX-4A and C-CTX-4B, formally known as gambiertoxins), which are produced by epiphytic and epilithic benthic dinoflagellates in the genus *Gambierdiscus*. These ciguatoxins are capable of trophic transfer by virtue of their lipo-

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philic properties, leading to their accumulation in consumers. In the Caribbean, the US Virgin Islands are classified as hyperendemic areas for CFP (Lange et al. 1992), indicating that poisonings are relatively common. Because CFP affects human health, the unique symptomology of this syndrome has been well documented. However, identifying the environmental conditions contributing to CFP remains challenging due to the multitude of potential influential factors and their interactions. Laboratory studies have assessed a suite of environmental factors such as light intensity, salinity, temperature and the effects these factors have on the growth and abundance of *Gambierdiscus* spp. (hereafter *Gambierdiscus* due to our genus-level observations) (Bomber et al. 1988, Morton et al. 1992). Observational field studies have sought to identify correlations between cell abundance and ambient nutrient levels, light intensity, water motion, temperature, and depth, with mixed and sometimes contradictory conclusions (Yasumoto et al. 1980, Carlson & Tindall 1985, Villareal & Morton 2002, Parsons & Preskitt 2007, Richlen & Lobel 2011). To our knowledge, however, there have been no observational studies investigating effects of grazing on *Gambierdiscus* cell abundance (either in the lab or field), nor have any field studies manipulated nutrient availability.

Gambierdiscus populations depend on growth rate, loss processes and carrying capacity. Factors reportedly contributing to the growth rate of *Gambierdiscus* include nutrient supply, light, and temperature. *Gambierdiscus* are photosynthetic dinoflagellates requiring light for growth, and are typically found in shallow waters. Optimal growth rates have been documented at temperatures of ~28°C in controlled laboratory studies, while peak abundances recorded in the field corresponded to temperatures of ~30°C (Bomber et al. 1988). *Gambierdiscus* also require nutrients for growth, and can utilize organic and inorganic nitrogen (Lartigue et al. 2009). Ambient nutrient levels observed in the field have not shown consistent correlations to *Gambierdiscus* abundances (Yasumoto et al. 1980, Carlson & Tindall 1985, Parsons & Preskitt 2007), and to date the nutrient source, type, and concentration that maximizes growth has not been determined.

Both abiotic and biotic factors contribute to the removal of *Gambierdiscus* cells from a location. The process by which cell loss occurs is an important but poorly described aspect of *Gambierdiscus* population dynamics. Abiotic disturbance events from water motion are potentially significant contributors to changes in *Gambierdiscus* populations, and investigators have reported an inverse relationship between turbulence and cell densities (Tindall & Morton 1998, Richlen & Lobel 2011). Herbivory is the biotic mode by which ciguatoxins enter the food web; this functional removal process may therefore be important in determining the distribution and abundance of *Gambierdiscus* on benthic structures, in addition to having human health implications. The effect of grazing on the abundance of *Gambierdiscus* is unknown, but the palatability and nutritional characteristics of the host algae (Cruz-Rivera & Villareal 2006), detritus (Purcell & Bellwood 2001), or rate of grazing (Carpenter 1986) will likely determine its impact. In addition, the inadvertent disturbances caused by the grazing activities of fish may facilitate the dislodgment and loss of *Gambierdiscus* cells from an area.

The depth that *Gambierdiscus* inhabits has profound effects on cellular growth and attachment in both shallow and deep-water environments. *Gambierdiscus* is a photosynthetic dinoflagellate whose growth is light dependent, and therefore growth is restricted at depths without sufficient light penetration. Conversely, growth may be inhibited in shallow waters where light levels are too intense, as *Gambierdiscus* have been shown to be intolerant of high light intensities in cultures (Morton et al. 1992, Villareal & Morton 2002, Bomber et al. 1988). Low salinities also adversely affect growth rates (Yasumoto et al. 1980, Bomber et al. 1988); therefore, growth may be inhibited in very shallow waters where surface layers are less saline due to less dense water from rainfall, rivers, groundwater, and runoff. Furthermore, shallow surface layers of water may not be thermally well mixed, potentially reaching high temperature extremes that could exceed *Gambierdiscus* metabolic maxima, slowing growth, inducing emigration, or causing mortality.

Despite laboratory investigations of light, nutrients, temperature and salinity on growth rates of *Gambierdiscus*, few studies have observed the impacts of these factors in a natural reef environment. Natural conditions often produce responses that are different from laboratory conditions and the interactions between factors can often lead to surprising results that are not predicted by uni-dimensional responses to a single factor. The purpose of this research was to investigate the interacting effects of depth, excess nutrients, and physical disturbances from grazing and water motion on *Gambierdiscus* abundance in natural populations.

Currently, there have been no experimental or observational investigations of the effects of grazing on *Gambierdiscus*. Here we sought to identify the

effects of grazing and disturbance by eliminating these pressures on host substrates. *In situ* nutrient availability was also manipulated to assess the importance of nutrient abundance and ratios on *Gambierdiscus* in natural systems. Our goal was to gain insight into the level of nutrients that are growth limiting, and how nutrient ratios affect *Gambierdiscus* cells in natural systems, both of which are needed to improve the accuracy of population dynamics models (Parsons et al. 2010). To understand better how the physical environment inherent with depth influences the abundance and distribution of *Gambierdiscus,* this study sampled at 20 and 10 m depth. This range in depth represents a significant portion of the shallow water reef environment in the US Virgin Islands.

We hypothesized that *Gambierdiscus* abundances are enhanced with a reduction in grazing rates, an increase in nutrient availability and depth (reduced water motion). Thus, abundance would be highest at depth (reduced water motion) and when substrates are ungrazed and nutrified; in contrast, abundance would be lowest in shallow waters (increased water motion) and when substrates are grazed and non-nutrified. Our experimental approach used standardized methodologies within the context of a novel manipulative field experiment to examine how multiple ecological factors influence the abundance and distribution of *Gambierdiscus*.

MATERIALS AND METHODS

Study area

This study was conducted on reefs surrounding St. Thomas, US Virgin Islands, a small Caribbean island located ~70 km due east of Puerto Rico (Fig. 1). Field activities were conducted over 74 days between 30 September and 13 December 2010 (treatments deployed at all sites on 29 October) at sites located on the southwest (leeward) side of the island. Experiments were carried out at 3 sites: (1) Perseverance (18° 20′ 45″ N, 64° 59′ 58″ W), (2) Flat Cay (18° 19′ 06″ N, 64° 59′ 27″ W), and (3) Saba (18° 18′ 11″ N, 64° 59′ 56″ W) (Fig. 1). Depths of ~20 and ~10 m were sampled within each location (exact depths in parentheses): Perseverance (18.9 m, 10.1 m), Flat Cay (19.8 m, 10.7 m), and Saba (22.9 m, 9.14 m). Each site consisted of ~10% hard coral cover, and a combination of hard bottom, sand patches, dead coral with algae, turf algae, and macroalgae.

Experimental tiles

The experimental substrate consisted of sandstone tiles with a coarse-grained abrasive surface, pre-cut to the dimensions of 10 cm^2 at a thickness of 1 cm,

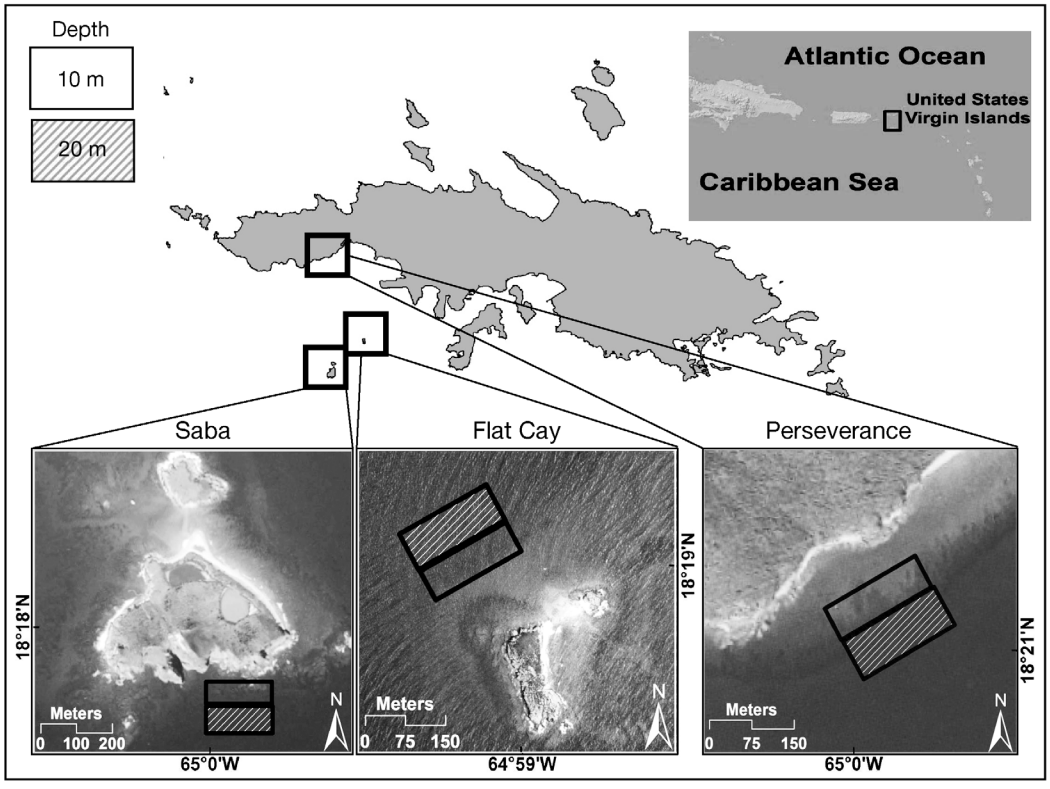

Fig. 1. Shallow (10 m) and deep water (20 m) sampling locations at 3 sites (Saba, Flat Cay and Perseverance) in St. Thomas, US Virgin Islands

and weighing approx. 200 g. Tiles were used to represent a natural substrate for *Gambierdiscus* colonization. All tiles were attached (using cable ties) to a 13 cm^2 zinc-coated galvanized wire mesh (no. 19 gauge) with a diagonal aperture of 1.7 cm. Tiles (n = 44) were arranged approx. 1 m apart within an 8 × 7 m area at each of the 6 sites, oriented lying flat with the wire mesh back on the benthos and the side for colonization facing the water's surface. Tiles were deployed for 30 d of *in situ* conditioning prior to study initiation in order to acclimate and establish a population of *Gambierdiscus*. After acclimation, 10 unmanipulated tiles serving as initial controls were collected at each site and depth and processed in the laboratory. Tiles were collected underwater and placed in Ziploc bags, with minimal handling to avoid disturbing the epiphytic community. This 30 d acclimation period was sufficient, since 100% of the initial control tiles were colonized by the dinoflagellate *Gambierdiscus* (presence and enumeration performed in the laboratory via light microscopy).

Experimental treatments: nutrients, caging, and partial caging

Following the initial control tile collection, tiles (n = 6 per treatment, per site) were each subjected to one of the 4 following treatments: (1) full caging, (2) full caging with nutrient enrichment, (3) partial caging, or (4) partial caging with nutrient enrichment. Ten tiles were left unmanipulated to serve as final controls, giving a total of 34 tiles per site. For the caging treatments, 72 full cages (13 cm^3) and 72 partial cages were constructed from the same zinc-coated galvanized wire mesh from which the bases were constructed and secured by cable ties to their wire mesh base. Cages excluded any grazer that could not pass through a diagonal aperture of 1.7 cm. Each partial cage consisted of a base, 2 sides (oriented as a corner to remain open to grazers), and a mesh roof to control for the effects of shading experienced by the full cage; all surfaces of the partial cage were 13 cm^2. The level of nutrients available to *Gambierdiscus* cells colonizing the experimental tiles was elevated using nutrient pouches containing Osmocote Smart-Release Plant food fertilizer (Sierra Chemical Company). This nutrient source has been used in a variety of *in situ* studies and can increase ambient nutrient concentrations by several-fold (Worm et al. 2000, Littler et al. 2006). The coated fertilizer is guaranteed to contain a total nitrogen (N) concentration of 19% (10% ammoniacal nitrogen, 9% nitrate nitrogen), available concentration of 6% phosphate (P_2O_5), and 12% soluble potash (K_2O). To retain the Osmocote pellets, nutrient diffusers were fabricated from porous plastic window screen material with a mesh size <1 mm into 72 (10.5 × 13.5 cm) pouches and each were given 160 g (30g N, 9.6g P, 19.2g K) of fertilizer pellets. The nutrient pouches were attached under the wire mesh base of the tiles of 36 full cages and 36 partial cages prior to field deployment with cable ties affixed on the edges. Tiles subjected to nutrient enrichment were placed above the nutrient pouch and secured to the cage or partial cage with cable ties; therefore, these pouches did not shade the tile surface. All experimental treatments lasted ca. 30 d.

Sample processing and *Gambierdiscus* spp. enumeration

Sample tiles were transported to the laboratory at ambient temperatures, placed in a clean 8 l container with 1.5 l of filtered seawater (FSW) (1.5 µm GFF vacuum filtered) and all surfaces of the tile scraped using a soft bristle brush (additional water used when necessary). Samples were filtered through a 202 µm nitex sieve to remove particles larger than typical *Gambierdiscus* sizes (42–150 µm transverse diameters) and onto a second 20 µm sieve to capture *Gambierdiscus* and particulate matter. Material retained on the 20 µm sieve was backwashed with FSW into a 15 ml conical centrifuge tube to a target volume of 10 ml. Samples were then preserved with the addition of formalin, to create a 4–5% solution, and the particulate volume in each tube was recorded as the amount of visible material settled after a minimum 48 h period.

Processed samples were gently shaken before a 1 ml aliquot was placed on a Sedgewick Rafter counting slide. Contents were analyzed for *Gambierdiscus* abundance (genus only) using an Eclipse E400 light microscope (Nikon) at 10× magnification. Cells were identified using photomicrographs and line drawings from Faust & Gulledge (2002), Richlen et al. (2008), and Litaker et al. (2009). Counts were expressed as a density of cells per cm^2.

Water motion measurements

Water motion was measured at each sampling site using the 'clod-cards' technique developed by Doty (1971). The clod cards were deployed in groups of 10 at all sites on 30 September (Period A), 2 December

(Period B), and 12 December (Period C) for approx. 24 h. In addition to the 10 exposed clod cards, 3 control clods were placed at the same time and location in a 19 l bucket with a sealed lid to eliminate the effect of water motion, while simultaneously exposing them to the same salinity and temperature regime as the exposed clod cards (Jokiel & Morrissey 1993). Weight loss over time for each set of experimental clod cards was divided by the average weight loss of their corresponding control clod cards to obtain the diffusion index factor (DIF) for each treatment. The DIF translates into a rate that is comparable across sites, as well as to different clod card batches and DIF values reported from other studies (Doty 1971). The control set of clod cards for Saba at 10 m depth on 30 September was lost, therefore the values from both Perseverance and Flat Cay at 10 m were averaged (equaling 1.37 ± 0.75, mean ± SD) and used to represent the lost Saba control.

Nutrient analysis

To determine ambient nutrient concentrations, water samples were collected at all sites and depths at multiple time points over a 4 month period in 2010, which included the tile deployments on 30 September at all 3 site locations, the treatment deployments on 29 October at Perseverance, and when tiles were collected at the end of the experiment on 2 December (Perseverance and Flat Cay), and 13 December (Saba). To determine the experimental nutrient enrichment of the tiles (above ambient concentration), water samples were taken approx. 5 cm above the surface of these tiles at the start (29 October) and conclusion (13 December) of the experiment at Perseverance and Saba. These samples were collected using a sterile 60 ml plastic syringe and immediately filtered on the boat using a syringe tip Sterivex GP 0.22 µm filter. Samples were then put on ice for transport to the lab where it was frozen at −25°C until analysis. Ambient water samples were collected 1 m above the reef surface using sterile Whirlpak bags, and upon returning to the boat were processed in the same way as the nutrified samples. All samples were sent frozen to the Nutrient Analytical Facility at the Woods Hole Oceanographic Institution (Woods Hole, MA) where they were analyzed for silicate, NH_4^+, PO_4^{3-}, and NO_2+NO_3 using a Lachat QuickChem 8000 according to standard protocols. Silicate was used as a control and not manipulated in this study. All collection and processing was completed while wearing nitrile exam gloves.

Fish surveys

Fish community structure and biomass were measured at both depths (10 and 20 m) and at all sampling locations, based on the methods established by Sandin et al. (2008). Each fish survey (n = 19) was recorded *in situ* by a fish census diver who had been trained and tested annually for size estimation accuracy using floating plastic models. Surveys were conducted during the day between November (when the treatments were deployed) and December (when the tiles were collected). At each location, 3 quadrats (5 × 2 m) were sampled (except at Perseverance at 10 m, where 4 quadrats were sampled) within and around the project study site locations; a total of 19 quadrats were surveyed. During the course of each 13 min survey, all fish observed within the quadrat were identified to species level, counted, and their total length estimated to the nearest centimeter and data recorded on waterproof paper. Surveys consisted of water column and benthic components, based on species activity patterns (i.e. swimming in water column or site-attached). The quadrat was set 1 min prior to the start of recording the survey to allow sufficient time for the fish to acclimate to the presence of the quadrat, and during this time the temperature was recorded and visibility at the site estimated. After the acclimation period, the water column survey was conducted, consisting of a stable 1 min visual scan for all (non-site attached) fish swimming in the water column; this visual scan method was conducted at 3 time points throughout the 13 min survey: between minutes 0–1, 6–7, and 12–13. During the water column visual scan, large schools (>25 ind.) of brown chromis *Chromis multilineata*, blue chromis *Chromis cyanea*, and creole wrasse *Clepticus parrae* were, at times, difficult to single out for accurate length estimates, therefore, an average size estimate was made and the number of individuals recorded. The second part of the survey included benthos scans that were conducted during intervals between the water column visual scans between minutes 1–5 and 7–12. All observed site-attached fish in half of the quadrat were recorded during the first benthos scan (minutes 1–5), and all site-attached fish in the remaining half of the quadrat were surveyed during the second benthos scan (minutes 7–12).

The length of each fish observed during the survey was estimated to the nearest centimeter and entered into a size category bin (0–5, 6–10, 11–20, 21–30, 31–40, and >40 cm). The average value of these size categories was used to represent all individuals in their respective category, i.e. tallied fish in the

6–10 cm category would be represented by the average length in that category, in this case 8 cm. Only 4 individual fish (2 *Aulostomus maculatus* and 2 *Sphyraena barracuda*) were in the >40 cm category and were given the value of 50 cm. Total lengths were converted into biomass density estimates based on published weight-length relationships in Sandin et al. (2008) and available online at www.fishbase.org. The allometric function $M = \alpha L^{\beta}$ was used, where M is the mass of the fish (g), L represents fish standard length (cm), in addition α and β are species-specific constants. Species biomass estimates per survey replicate were made by taking the mid-point value of the length bin, calculating the biomass of individual fish, and finally summing all individual fish values for each survey replicate. If the fish was recorded during a water column scan then one-third of its biomass was counted (i.e. the average of 3 replicate 1-min scan samples).

Final quadrat biomass estimates were calculated as the sums of all site-attached fish plus the calculated average mobile fish count biomass. Within species, biomass was combined for each replicate survey and then divided by the number of surveys conducted at that site. This average biomass for each species at each site was divided by the area of the quadrat (10 m^2) to obtain a biomass estimate in g per m^2. Fish were then organized into 5 trophic groups based on primary diet composition: piscivore, planktivore, herbivore, detritivore, and omnivore.

Statistical analyses

A 3-way ANOVA model was used to investigate the effects of the 3 main treatments: grazing, elevated nutrients, depth (water motion), and their interactions on the density of *Gambierdiscus* on tile surfaces and the fit of the whole statistical model. Data were first tested for homogeneity of variance and normality, and transformed when necessary. *Gambierdiscus* counts were log transformed. Sites were pooled because we found no significant difference in *Gambierdiscus* abundances (1-way ANOVA; $F = 0.16$, df = 2, p = 0.85) between sites at the start of the experiment (Saba = 2.0 ± 0.33 cells cm^{-2} [mean ± SE], Flat Cay = 2.3 ± 0.34 cells cm^{-2}, Perseverance = 2.1 ± 0.35 cells cm^{-2}), and no significant difference (1-way ANOVA; $F = 1.52$, df = 2, p = 0.23) between final control tiles by site at the conclusion of the experiment (Saba = 3.8 ± 0.46 cells cm^{-2}, Flat Cay = 2.7 ± 0.46 cells cm^{-2}, Perseverance = 2.8 ± 0.64 cells cm^{-2}). The partially caged tiles were used to represent the ambient condition (non-nutrified) tiles in the 3-way ANOVA because we found no difference (one-way ANOVA; $F = 2.36$, df = 1, p = 0.13) between the unmanipulated control tiles collected at the conclusion of the experiment and the partially caged tiles (partial cage = 4.1 ± 0.47 cells cm^{-2}, final control = 3.2 ± 0.39 cells cm^{-2}). We also found no difference in *Gambierdiscus* abundance between the nutrient treatments; similar cell densities were observed in both caged (9.5 ± 1.8 cells cm^{-2}) and caged with nutrients (9.6 ± 1.8 cells cm^{-2}) ($F = 0.001$, df = 1, p = 0.97), and partially caged (4.1 ± 0.54 cells cm^{-2}) and partially caged with nutrients (3.8 ± 0.55 cells cm^{-2}) ($F = 0.129$, df = 1, p = 0.72). Therefore, when testing the effects of caging, these nutrified tiles were combined for their caging treatment effects. Since caging was found to be significant following the test for main effects, a 1-way ANOVA with Bonferroni correction for multiple tests was run to compare the caged treatments to identify further if a site was heavily influencing the measured caging effect. We found a strong site effect on the results of the caged treatments at Saba but the caging results were significant with or without the addition of data generated at Saba. Post hoc comparisons were performed using Tukey HSD tests.

We observed a general increase in epilithic algae, including turfs and cyanobacteria in the caged treatments. We were unable to measure the algal surface area, so we measured the particulate volumes that accumulated in the collected samples, as epilithic algal standing crop and particulate sediment load have been found to be significantly positively correlated (Purcell & Bellwood 2001). To investigate this observation, a 3-way ANOVA model was used to determine any effects of the main experimental factors on the volume of particulates. To determine if there was a relationship between the particulate volume and the cell counts, a linear regression was performed between cell abundance per area and particulate volume. We also conducted a separate 3-way ANOVA model test with the 3 types of treatments as independent variables, but with cell counts standardized by particulate volume rather than tile surface area. The reasoning was that if particulate matter were a proxy for the abundance of epiphytic algae, then standardizing to particulate volume would have the effect of removing the impact of increasing colonizable surface (epiphytic algae) from the treatments. Post hoc comparisons were performed using Tukey HSD tests.

The water motion measurements were square root transformed and then analyzed using a 3-way ANOVA for the effects and interactions of site, depth,

Table 1. Results of 3-way ANOVAs to assess the effects of depth, caging, and nutrients on *Gambierdiscus* abundance and the accumulated volume of particulates on final experimental tiles. *Gambierdiscus* abundance was standardized by tile area (with all sites pooled for treatment effect) or by particulate volume. **Bold** values indicate significance

Effect test	*Gambierdiscus* abund. (tile area)			*Gambierdiscus* abund. (particulate vol.)			Particulate volume		
	df	*F*	p	df	*F*	p	df	*F*	p
Whole Model	7,84	2.999	**0.006**	7,131	1.2	0.307	7,180	9.397	**<0.0001**
Depth	1,91	2.692	0.103	1,137	0.918	0.34	1,186	0.361	0.5492
Caging	1,91	15.2	**0.0002**	1,137	3.515	0.063	1,186	49.59	**<0.0001**
Nutrients	1,91	0.236	0.628	1,137	2.436	0.121	1,186	9.8	**0.0022**
Depth × Nutrients	1,91	1.261	0.264	1,137	0.018	0.895	1,186	0.183	0.6698
Depth × Caging	1,91	1.204	0.275	1,137	0.02	0.889	1,186	5.212	**0.0241**
Nutrients × Caging	1,91	0.014	0.905	1,137	0.144	0.705	1,186	0.439	0.5087
Depth × Nutrients × Caging	1,91	0.52	0.472	1,137	1.281	0.26	1,186	0.743	0.3903

and period. A repeated measures ANOVA was not used because the clod cards were created independently (standardized by methodology and their own controls) and periods of water motion were considered independent due to the natural variability in current direction, periodicity, and wind driven wave direction present at each site. Post hoc comparisons were performed using Tukey HSD tests. A 2-way ANOVA was also performed to model the interactions of site and depth for the ambient water samples collected, with post hoc comparisons again performed using Tukey HSD tests. All nutrient measurements met the assumptions of ANOVA and were not transformed, except NH_4^+ data, which were log transformed.

The fish survey data did not meet the assumptions of an ANOVA; therefore, a Kruskal-Wallis test was performed to compare differences in mean biomass per area. Statistical analyses were performed using JMP software (v.9, SAS).

RESULTS

Effect of depth, caging and excess nutrients

A strong effect of caging was observed with a greater than 2-fold increase in the abundance of *Gambierdiscus* compared to partially caged treatments (cage = 9.5 ± 0.94 cells cm^{-2} [mean ± SE], n = 72, partial cage = 4.0 ± 0.97 cells cm^{-2}, n = 67) (Table 1, Fig. 2). There was also a significant site effect on the mean abundance of *Gambierdiscus* on caged substrates (F = 14.3, df = 2, p < 0.0001). The disparity between the caged substrates and the substrates open to grazing was most evident at Saba, where the abundance of *Gambierdiscus* on caged tiles averaged 18 ± 1.85 cells cm^{-2} compared to averages of 4.9 ± 1.56 cells cm^{-2} for partially caged tiles and 3.8 ± 1.7 cells cm^{-2} for control final tiles (i.e. cells increased by 374% between partially caged and caged treatments). Average cell counts at Saba for the caged treatments (18 ± 1.85 cells cm^{-2}) were significantly different (F = 14.3, df = 2, p < 0.0001) from Perseverance (6.2 ± 1.85 cells cm^{-2}) and Flat Cay (4.8 ± 1.85 cells cm^{-2}), which were not significantly different from each other. However, results for the effect of caging on the abundance of *Gambierdiscus* were robust across sites, as caging was significant even when Saba was excluded from the analysis (F = 5.91, df = 1, p = 0.0171). There were no detectable effects of depth or nutrients on *Gambierdiscus* abundance per cm^2 (Table 1), even at the high levels of nutrient enrichment achieved by nutrient addition (up to a measured peak of 53,833% above ambient conditions), nor were there any significant interactions between treatments on the abundance or distribution among sampling depths.

Gambierdiscus cell density and sample particulate volume showed a positive linear (cell density = −0.89265 + 3.77627 × particulate volume) and significant relationship (r^2 = 0.233, p < 0.0001). The amount of settled particulates in the 15 ml conical tubes differed significantly by treatment (Fig. 2). Caging had a strong effect on the accumulation of particulates (Table 1), although there was a significant difference between cages, as cages without nutrients had significantly more particulate matter than cages with additional nutrients (Fig. 2). Caging with nutrients did have significantly more particulates than any other non-caged tile treatment including partially caged, partially caged with nutrients, or final control tiles (Fig. 2). Tiles without full cages were not signif-

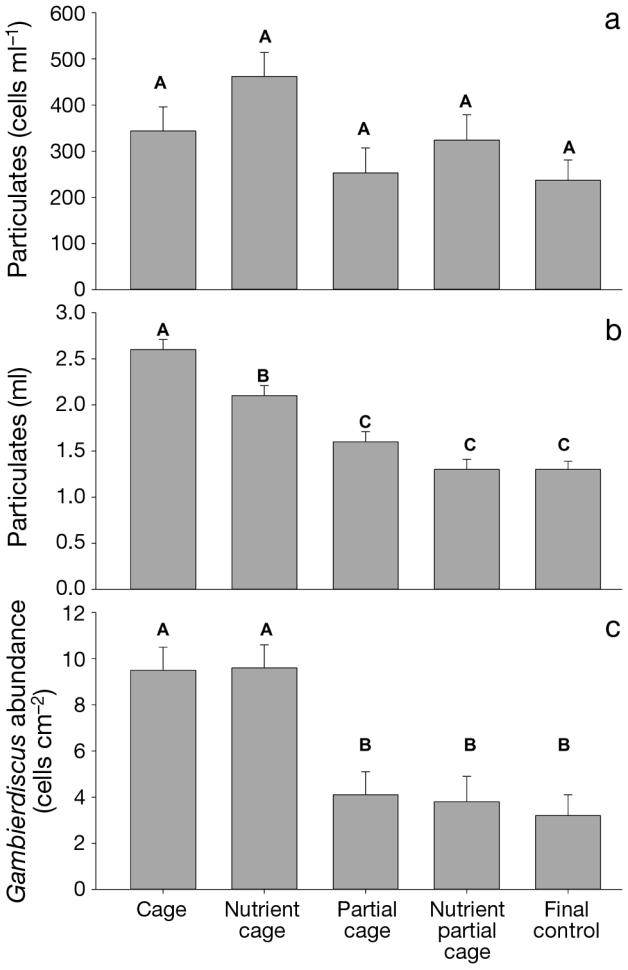

Fig. 2. Mean (+SE) (a) *Gambierdiscus* cells per volume of particulates in original units, (b) final volume of particulates on experimental tiles, and (c) final abundance of *Gambierdiscus* cells per tile area in the different experimental treatments. Data was log transformed for analysis. Treatments not sharing an uppercase letter are significantly different (Tukey's HSD; $p < 0.05$)

icantly different from one another (Fig. 2). Because of the positive influence of caging on particulate volume and the positive influence of particulate volume on *Gambierdiscus* abundance, cell counts were standardized to particulate volume. When cell counts were standardized to particulate volume and log transformed there was no difference between treatments (Table 1, Fig. 2). The caging treatment did influence the volume of particulate matter, thus leading to a significantly higher number of cells per area. Although the number of cells by particulate volume was not significantly different by treatment, nutrified treatments had on average 32% more cells per ml of particulate matter than their non-nutrified counterparts (Fig. 2).

Water motion measurements and nutrient analysis

Water motion measurements were significantly different among sites, depths, and sampling periods (Table 2, Fig. 3). There was a significant interaction at the site × depth × period level, indicating a variable pattern of water motion in time and space (Table 2). Diffusion index factor (DIF) at Saba (5.0 ± 0.23) was significantly higher ($F = 12.1$, df = 2, $p < 0.0001$) than at both Flat Cay (4.1 ± 0.23) and Perseverance (3.4 ± 0.23), and Flat Cay had a significantly higher DIF than Perseverance ($F = 7.28$, df = 1, $p = 0.008$). Saba, at 10 m depth, had the highest measured mean DIF (9.7), which occurred in the first measurement period during a period of wave-generated turbulence (Period A: mean wind speed = 7.2 m s^{-1}, direction = 92°). When wind speed was similar and direction was such that fetch length was restricted due to shielding from St. Thomas, this site was not different from the other sites (Period B: mean wind speed = 6.7 m s^{-1}, direction = 51°). The lowest mean diffusion factor (1.9) measured was from the shallow location at Perseverance. Sampling period A had the largest DIF recorded (5.4 ± 0.21) and was significantly different from the other sampling periods B and C ($F = 25.7$, df = 2, $p < 0.0001$), while sampling periods B (3.5 ± 0.22) and C (3.6 ± 0.22) were not significantly different.

Throughout the treatment period, water samples collected above nutrified plates had levels of NO_2+NO_3, and PO_4^{3-} that increased by 1289 to 53,833% above ambient nutrient levels (Table 3). All levels of manipulated nutrients declined over the course of the study, except for NH_4^+ which showed an increase in concentration over time, starting at 0%, and in the final measurement was 1358% higher than ambient samples. The initial measurement col-

Table 2. Results of 3-way ANOVA model comparing water motion (clod cards) by depth, site, period, and interactions among these factors. Water motion data were square root transformed. All water measurements were significantly different among sites, depths and sampling periods

Effect test	df	*F*	p
Whole Model	17,161	73.5	<0.0001
Site	2,175	84.5	<0.0001
Depth	1,176	123	<0.0001
Site × Depth	2,175	24.7	0.0002
Period	2,175	160	<0.0001
Site × Period	4,173	15.4	<0.0001
Depth × Period	2,175	135	<0.0001
Site × Depth × Period	4,173	63.3	<0.0001

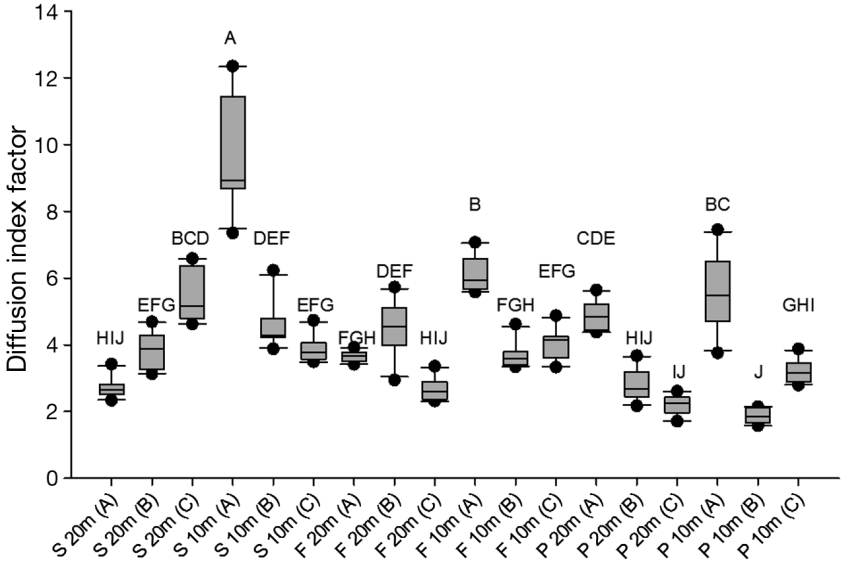

Fig. 3. Diffusion index factors (see 'Materials and methods: Water motion measurements' for details) for clod cards organized by site (S = Saba, F = Flat Cay, P = Perseverance), depth (10 and 20 m), and sampling period (24 h): A = 30 September, B = 2 December, and C = 12 December. Boxes indicate median and quartile ranges, whiskers delineate full ranges, black dots are maximum and minimum values. Groups not sharing an uppercase letter are significantly different (Tukey's HSD; $p < 0.05$)

lected above nutrified plates was taken at the time of deployment; thus, it is possible the NH_4^+ nutrient source needed time to dissolve in order to disperse. Ambient water sample nutrient concentrations were similar across sites, but ammonia did show significantly higher concentrations at the deeper (20 m) study areas (Fig. 4). Phosphate concentrations were generally very low, but Saba had significantly higher levels of PO_4^{3-} than Flat Cay (Fig. 4). Levels of the unmanipulated nutrient silicate, serving as a control, remained stable over the course of this study across sites, depths, periods, and nutrient manipulation treatments (Fig. 4).

Fish surveys

Temperatures recorded during fish surveys conducted in November and December ranged between 25.5°C and 27.2°C, and visibility ranged from 9.1 to 21.5 m. There was a statistically significant difference in the biomass per area between sites (Kruskal-Wallis; $\chi^2 = 6.47$, $F = 4.09$, df = 2, $p = 0.018$). The average total biomass among fish surveys, across sites and depths ranged from 41 to 192 g m^{-2} (mean = 98 g m^{-2}) (Fig. 5). Flat Cay (mean total biomass = 318 g m^{-2}) had twice the total biomass per area as Saba (134 g m^{-2}) or Perseverance (135 g m^{-2}). Approximately 35% of the total sum of mean biomass across all sites and depths was composed of piscivores (33.5 g m^{-2}), while herbivores comprised 19% of the total sum of mean biomass (18 g m^{-2}). The ratio of herbivore to piscivore biomass (g m^{-2}) for each site was 5:4 at Saba, 4:13 at Flat Cay, and 2:3 at Perseverance. Saba, the only site with a >1 herbivore to piscivore biomass ratio, and which had a 374% increase in cell abundance when herbivory was excluded, exhibited the largest difference between treatments open and closed to grazing among sites.

DISCUSSION

This investigation employed a novel experimental approach to identify and characterize the environmental factors influencing the dynamic balance be-

Table 3. Mean (±SE) nutrient concentrations (µM) in water samples collected near nutrified plates (with nutrient enrichment) and ambient plates (without additional nutrients) at the start (30 September 2010), on treatment deployment (29 October 2010), and at the end of the experiment (2 and 13 December 2010). The % increase is the positive difference between nutrified water samples and their respective ambient water samples (treatment deployment with and without nutrients and experiment end with and without nutrients). Samples collected at 20 and 10 m were averaged for each site. N = number of samples collected

Time	Site	N	NH_4^+	Silicate	PO_4^{3-}	NO_2+NO_3
Start: ambient	All sites	12	0.24 ± 0.08	2.82 ± 0.28	0.08 ± 0.01	0.05 ± 0.00
Deployment: ambient	Perseverance	4	0.59 ± 0.28	3.68 ± 0.21	0.11 ± 0.01	0.15 ± 0.05
Deployment: nutrients	Perseverance	4	0.24 ± 0.11	3.6 ± 0.32	8.0 ± 3.8	80.9 ± 40.4
Deployment: % increase			0	0	7250	53 833
End: ambient	All sites	12	0.94 ± 0.24	3.16 ± 0.34	0.09 ± 0.01	0.24 ± 0.02
End: nutrients	Saba	4	13.7 ± 6.51	2.71 ± 0.42	1.25 ± 0.58	12.1 ± 5.85
End: % increase			1358	0	1289	4942

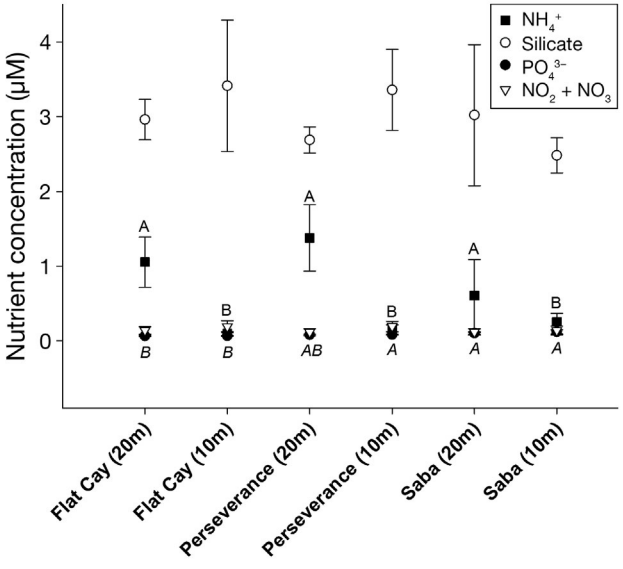

Fig. 4. Mean (±SE) ambient water sample nutrient concentrations for NH_4^+, silicate, PO_4^{3-}, and NO_2+NO_3 at different sites (Flat Cay, Perseverance, and Saba) and depths (10 and 20 m). Sites not sharing an uppercase letter are significantly different (Tukey's HSD; $p < 0.05$). Only NH_4^+ and PO_4^{3-} had significant differences among sites, results for PO_4^{3-} are italicized

tween cellular growth and loss of *Gambierdiscus* populations *in situ*, as well as the interactions among these factors. We hypothesized that *Gambierdiscus* population abundance would be elevated by increasing the availability of nutrients, at increased depth (reduced water motion), and when biological grazing and disturbance pressures were reduced. Our field studies did not detect any significant effect of depth or nutrients on populations of *Gambierdiscus*, however, *Gambierdiscus* cell densities were up to 374% higher when the effects of grazing were limited (i.e. by excluding all grazers >1.7 cm). The significant effect of grazing observed in this study suggested that changes in feeding selectivity and grazing rates of herbivores and detritivores might have a large impact on the areal density of *Gambierdiscus* in natural systems. Thus, the management of grazer populations in ciguatera endemic areas may ultimately have human health implications.

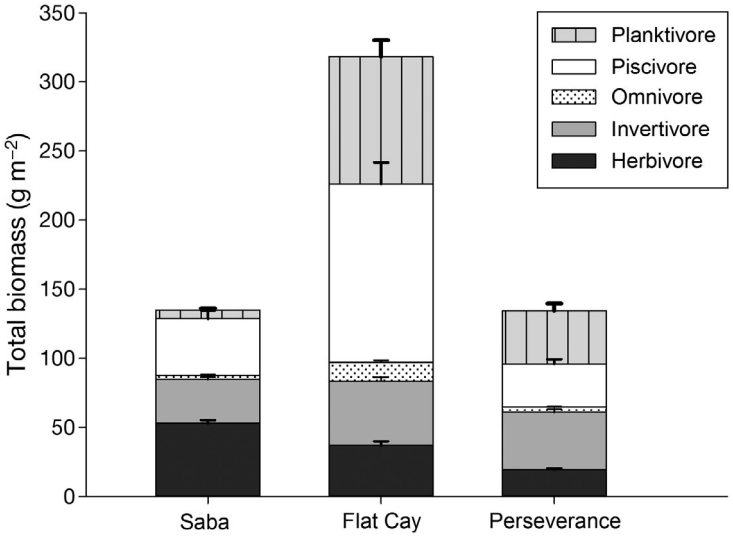

Fig. 5. Biomass (+SE) of fish community and constituent trophic groups at Saba, Flat Cay, and Perseverance. Total stacked bars represent a combined overall biomass for each site

Effects of grazing

Grazing resulted in significant decreases in the abundance of *Gambierdiscus* cells attached to the experimental substrates. The inverse was true when grazing was prevented by caging. This suggests that changes in grazing rates on reef substrates may have large impacts on the areal density of *Gambierdiscus* in natural systems. Since *Gambierdiscus* is not exclusively benthic, and can swim freely, potentially swimming from ungrazed caged surfaces to grazed, this relationship between benthic grazing and *Gambierdiscus* abundance was not obvious. The processes underlying these abundance changes are complex, and may be due to such factors as the lower occurrence of benthic disturbance and rate of ingestion, or the increased availability of substrate (epilithic algae and particulates) for attachment and growth. Through conducting the fish surveys, we also have a preliminary understanding of the direct and indirect impacts of grazing by the herbivore community on *Gambierdiscus* abundance. Total coral reef herbivores have been observed to remove more than 90% of the biomass produced daily by palatable marine macroalgae, with grazing intensity rates of 20 000 to 156 000 bites m^{-2} d^{-1} by herbivorous fishes on shallow water reefs of the US Virgin Islands (Carpenter 1986). Consequently, the grazing activities of these fish would either result in the displacement of *Gambierdiscus* cells by ingestion, or disturbance from grazing impacts on their host substrate. Presumably, disturbed cells would not necessarily be lost from the system, potentially settling on other substrates. This could possibly lead to ungrazed surfaces becoming a sink for cells displaced from nearby disturbances, a po-

tential explanation for the increased cell abundance on ungrazed treatments. Regardless of the mechanisms (e.g. ingestion, protection, disturbance, immigration, and increased substrate) underlying the observed decline or increase in *Gambierdiscus* from the grazing open or closed treatments, this study shows that grazing (and its related disturbances) is clearly a significant and quantifiable factor that influences the areal density of *Gambierdiscus* cells on coral reefs.

We observed a general increase in epilithic algae, including turfs and cyanobacteria, in the caged treatments. This proliferation of epilithic algae effectively enlarged the available substrate for colonization, and consequently may have increased the carrying capacity of the caged tiles for *Gambierdiscus* cells. While we were unable to measure the surface area of algae attached directly to the experimental substrates, we measured the particulate material volume that accumulated on the tiles. This particulate volume served as a proxy for the epilithic algae community standing crop, since the epilithic algal standing crop and particulate sediment load have been found to be significantly positively correlated (Purcell & Bellwood 2001). The particulate volume was significantly higher in caged treatments, suggesting a greater abundance of epilithic algae (Table 1, Fig. 2). Furthermore, the density of cells and particulate sediment volume were positively and significantly related. This may not always be the case as an ungrazed surface can be a source of emigration when *Gambierdiscus* swim, and the increase in particulates accumulated within the epilithic algal matrix (EAM) of an ungrazed surface may not always correlate with an increase in *Gambierdiscus* abundance. *Gambierdiscus* has been shown to persist in sediment (Faust 1995), and Parsons & Preskitt (2007) observed a preference of microfilamentous algae as a host habitat. These 2 components are part of the EAM, which is a primary food source for many herbivorous reef fishes (Wilson et al. 2003). These results suggest that the processes that led to the accumulation of particulates (e.g. exclusion of grazing disturbances, increased epilithic algal biomass) were important contributors to the increased *Gambierdiscus* abundance observed in the caged treatments. Thus, grazing may indirectly influence *Gambierdiscus* abundance by controlling the availability of substrate for colonization. Future experiments that explicitly vary the levels of disturbance and EAM could determine which components of grazing (i.e. ingestion, disturbance, or substrate availability) most strongly influence the distribution and abundance of *Gambierdiscus*.

Effects of water motion, depth, light, and nutrient enrichment

Neither depth nor water motion were associated with significant differences in the abundance of *Gambierdiscus* at our sampling sites, indicating that the method of attachment or shelter utilized by *Gambierdiscus* was sufficient to withstand the water motion and wave energy regimes experienced at these depths across all sites and periods. These results differ from those reported by Richlen & Lobel (2011), which found a significant negative correlation between water motion, as measured by clod cards, and *Gambierdiscus* abundance at sampling sites in Johnston Atoll (Pacific Ocean). However, average water-motion values for their study sites were up to 3 times larger than those measured in the current study, which may explain this discrepancy. The relatively lower water motion recorded throughout this investigation and lack of effect on *Gambierdiscus* implies that the water motion experienced at Saba, Flat Cay, and Perseverance was apparently insufficient to dislodge, redistribute or otherwise remove substantial numbers of *Gambierdiscus*. Because we found no difference in populations of *Gambierdiscus* at 10 or 20 m, this depth range could be treated as one type of environment, or water motion regime, when sampling for *Gambierdiscus* at these sites. Future studies that employ a greater range of depth and water motion regimes could provide evidence to help identify where along the depth gradient significant changes in abundance occur.

Gambierdiscus has been shown to grow best in the laboratory at ~232 µmol photons m^{-2} s^{-1}, which corresponds to ~11% of noonday surface irradiance at mid-low latitudes (Bomber et al. 1988). Measurements of photosynthetially active radiation (PAR, 400–700 nm) were collected in November 2012 at Flat Cay and Saba under noonday, clear-sky conditions by Brewer (2013). PAR measured at depths of ~10 m at Flat Cay corresponded to ~31% of PAR measured 1 m below the surface. Data collected at Saba at a depth of ~22 m approximated 18% of the PAR levels taken 1 m below the surface. These measurements give an approximation of the light levels at Flat Cay and Saba for our sampling depths, and correspond to a range of light levels above the optimum (>11%) for *Gambierdiscus* in which little variation in growth rate would be expected (Bomber et al. 1988). We observed no difference between population abundances at the 2 depths sampled. Therefore, either a change in abundance due to light variability between depths was masked by a compensating factor or this difference in

light intensity failed to cause a measurable change in the abundance of *Gambierdiscus*.

The addition of nutrients (NO_2, NO_3, NH_4^+, and PO_4^{3-}) also had no measurable effect on *Gambierdiscus* abundance, indicating that these nutrients were not growth limiting for *Gambierdiscus* at ambient concentrations. This suggests that any growth restrictions to natural *Gambierdiscus* populations resulting from insufficient resources may be due to other factors (e.g. dissolved inorganic carbon, interactions with host substrates, or processes that disturb the aqueous boundary layer). The results of this study are similar to those reported by Parsons & Preskitt (2007), which concluded that ambient nutrient concentrations among sampling sites around the island of Hawaii had no measurable effect on the abundance of *Gambierdiscus*. However, the availability of nutrients and their relative ratios have been demonstrated to influence other intercellular activities; for example, toxin production has been shown to increase because of a higher N:P ratio (N:P ≥ 30:1) (Sperr & Doucette 1996). Species of *Gambierdiscus* co-occur in natural populations and it is unclear what role nutrient availability may play in determining the species composition of a habitat. Thus, while we saw no change in the abundance of *Gambierdiscus* with the addition of nutrients it is possible that they had an effect on individual cell toxicity or species composition.

Considerations for fisheries management

To our knowledge, this study is the first to directly measure the impact of herbivory on *Gambierdiscus*, although, the importance of plant–herbivore interactions and their influence on toxin uptake has been investigated through assessments of the relative palatability of algal hosts for *Gambierdiscus* (Lobel et al. 1988, Cruz-Rivera & Villareal 2006). The factors influencing the abundance of *Gambierdiscus* on the tile substrates appeared to be largely biologically driven via the actions of removal through grazing and disturbance. By restricting grazing and disturbance processes, we recorded a greater than 2-fold increase in the abundance of *Gambierdiscus* at our sampling sites. At Saba, the site with the highest herbivore biomass by a factor of 2, caged tiles had, on average, 374% more cells compared with the control tiles exposed to grazing. The herbivore to piscivore biomass ratio was 4:13 at Flat Cay and 2:3 at Perseverance and caged tiles had 74% and 68% more cells on average (respectively) than tiles open to grazing. When considering the different biomechanisms responsible for decreased *Gambierdiscus* abundance in the open grazing treatments, it is important to note that the cell ingestion and disturbance rates for each grazer class would be unique, and as such could be used to classify grazers by their level of ingestion or disturbance impact on *Gambierdiscus*. The management of reef herbivore populations could affect the per area abundance of *Gambierdiscus* on benthic surfaces by either utilizing or restricting a herbivore populations' ability to remove cells directly or the substrate *Gambierdiscus* requires. We hypothesize that if the predator–prey interaction between grazers and *Gambierdiscus* were to take place in isolation, increased grazing would at first raise toxin levels in fish. However, if grazing exceeds *Gambierdiscus* growth rates over time causing *Gambierdiscus* abundance to decline, the cells ingested per bite by a grazer should also decline, resulting in a decrease in consumed ciguatoxin concentrations per gram of herbivore flesh. Trap fishing is permitted at the sites surveyed in this study, therefore, these sites may be a good area to continue investigating the effects of anthropogenic disturbances to grazing via fishing and the subsequent potential interactions on *Gambierdiscus* abundance. The fisheries pressure currently employed at these sites may be affecting the significant differences in fish biomass per area observed at these study sites. What is not clear is whether a reduction in grazing and the resulting increase in cell abundance would translate into higher cell ingestion rates for other primary consumers or enhanced transfer of toxins to reef fishes, all of which depend on specific trophic transfer mechanisms. The importance of grazers in a ciguatoxin prevalent area, and how the management of their populations ultimately affects human health are subjects that remain largely unstudied.

Conclusions

St. Thomas is subject to heavy fishing pressures, and is also endemic for ciguatoxins. These toxins, given their bioaccumulative properties, are presumed to be present throughout the entire food web. This study showed that changes in feeding selectivity and grazing rates of reef fishes is clearly a significant and quantifiable factor that influences the areal density of *Gambierdiscus* cells on coral reefs; however, whether or not reduced grazing rates or disturbances translate into higher cell (toxin) ingestion rates is unknown. The only site with a >1 herbivore to pisci-

vore biomass ratio also had the greatest contrast between low *Gambierdiscus* populations on tiles open to grazing, and higher abundances on ungrazed treatments. *Gambierdiscus* cell density and sample particulate volume showed a positive and significant linear relationship, and caging had a strong effect on the accumulation of particulates. However, when we standardized cell counts to particulate volume there was no clear treatment effect on the abundance of cells per volume of particulates. Neither depth nor water motion were associated with significant differences in the abundance of *Gambierdiscus* at our sampling sites. The addition of nutrients (NO_2, NO_3, NH_4^+, and PO_4^{3-}) also had no significant effect on *Gambierdiscus* abundance. As grazing of *Gambierdiscus* introduces ciguatoxins to the food web, the mode and rate of toxin uptake has important implications for their accumulation and trophic transfer. Additional studies are needed to further identify and quantify the direct and indirect effects of grazing on *Gambierdiscus*, including species-specific grazing mechanisms, and to determine the importance of algal host palatability. This study clearly identifies the importance of grazers in ciguatera endemic areas, and raises the question of how management of their populations affects the prevalence of toxicity in the food web.

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