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Original Article

Evaluation of 24-h screen deployments as a standardized platform to monitor *Gambierdiscus* populations in the Florida Keys and U.S. Virgin Islands

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ABSTRACT

Anchored mesh screens have been suggested as a standardized approach to monitor the cell abundances of epiphytic dinoflagellates in benthic habitats, including toxigenic members of the *Gambierdiscus* genus responsible for ciguatera poisoning (CP). Here we deployed screens for 24h at eight sites in the Florida Keys and St. Thomas (US Virgin Islands) to evaluate their performance relative to the traditional method of assessing *Gambierdiscus* abundance in which cell counts are normalized to wet weight of host algae. The 30-month study (April 2013 – August 2015) involved monthly sampling at sites where screens were suspended at near-bottom locations for a 24h period and retrieved, with concurrent collections of macrophytes; including *Halimeda, Laurencia*, and *Thalassia* in the Florida Keys, and *Dictyota* in both regions. *Gambierdiscus* cells were identified and enumerated in the screen and macrophyte samples, and several regression techniques were evaluated (linear regression using untransformed and log-transformed data; negative binomial distribution (NBD) regression) to determine how well the screen-derived data could estimate algal cell concentrations on the host algae. In all cases, the NBD models performed the best based on Akaike Information Criteria values, although 38% of the regressions were not statistically-significant, including all of the St. Thomas sites. The r^2 values were all < 0.75 and averaged 0.36, indicating relatively poor fit of the screen data. False negative results (regression models underestimating actual cell abundances) were common occurrences, ranging from 5 to 74% of the scenarios tested. In summary, these results indicate that 24h screen deployments do not appear to be consistent in all situations. Caution is therefore needed when considering 24h screens as a standardized monitoring approach for quantifying *Gambierdiscus* population dynamics across geography and ecosystems. Furthermore, neutral (artificial) substrates may not adequately capture either the host preference or palatability that likely influence the initial vector of toxin incorporation in the food web via herbivory on these macrophytes.

1. Introduction

Researchers are making considerable progress in developing methods to study and monitor the toxigenic members of the benthic dinoflagellate genus, *Gambierdiscus* that are responsible for ciguatera poisoning. Revision of the genus in 2009 (Litaker et al. 2009) has led to a rapid increase in new species descriptions, most recently *G. holmesii* and *G. lewisii* (Kretzschmar et al. 2019) from the Great Barrier Reef. Molecular tools are being developed to identify *Gambierdiscus* species in field samples, including qPCR methods (Vandersea et al. 2012; Nishimura et al. 2016), RFLP assays (Lozano-Duque et al. 2018; Lyu et al. 2017), metabarcoding (Smith et al. 2017), and FISH-based probes for identification and quantification (Pitz 2016; Pitz et al. 2021). Research is also progressing towards the identification of the most toxigenic *Gambierdiscus* species, which to date appear to be *G. polynesiensis* in the Pacific Ocean (Chinain et al. 2010), *G. excentricus* in the Atlantic Ocean, and both *G. excentricus* and *G. silvae* in the Caribbean Sea (Litaker et al. 2017; Robertson et al. 2018).

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Standardized sampling methods have also been proposed, including suction-based devices (Lewis et al. 1994; Parsons et al. 2010) and the use of artificial substrates (Caire et al. 1985; Tester et al. 2014; Parsons et al. 2017; Fernández-Zabala et al. 2019). These advances have paralleled a dramatic expansion in ciguatera research, increasing from an average of 20 (1998-2002) to 59 (2016-2020) publications per year over the last two decades (based on a literature search for *Gambierdiscus* in the Clarivate Analytics Web of Science database). With the rapid development of multiple methods to identify and monitor *Gambierdiscus* populations (and their toxicity), the time has come to field test these methods to assess their practicality, accuracy, and effectiveness, particularly when proposed as a standardized method for broader adoption by the research community (Tester et al. 2014; Berdalet et al. 2017). This is critical in today's climate, given the concern with the "reproducibility crisis" in science (Hossenfelder 2017); will these tools perform as advertised when used in other regions by other researchers?

This study builds upon previous research that evaluated the use of neutral (artificial) substrates to monitor *Gambierdiscus* and other benthic dinoflagellate populations (Tester et al. 2014; Jauzein et al. 2016; Parsons et al. 2017; Jauzein et al. 2018; Fernández-Zabala et al. 2019). Artificial substrates are attractive because they eliminate concerns regarding the wet weight-to-surface area variability inherent among the different macroalgae morphologies (i.e., should *Gambierdiscus* cell densities be reported as cells g^{-1} wet wt or cm^{-2}?). This variability was well exemplified in the study of Lobel et al. (1988), who demonstrated that *Gambierdiscus* cell abundances were greater on *Dictyota* when data were reported as cells g^{-1} wet wt, but were greater on *Galaxaura* when calculated as cells cm^{-2}. Artificial substrates are also neutral, which eliminates the potential role of host preference that may influence *Gambierdiscus* densities (either increasing or decreasing their likelihood of settling on a particular host species; Rains and Parsons 2015). Evidence of this phenomenon (host preference), however, is mixed (Parsons et al. 2017). Artificial substrates also produce cleaner samples for molecular analysis (Tester et al. 2014) and can improve precision in monitoring programs through strategic deployments of artificial substrates in time and space (Fernández-Zabala et al. 2019). Lastly, artificial substrates theoretically provide a constant sampling platform across seasons and sites (Tester et al. 2014; Berdalet et al. 2017; Jauzein et al. 2018; Fernández-Zabala et al. 2019), eliminating the occurrences in which a particular host macrophyte is absent or in variable abundance annually, which can greatly hinder monitoring efforts (i.e., missing data, use of a different host species which may provide different cell densities).

Tester et al. (2014) proposed the use of 24h screen deployments as a standardized method for monitoring *Gambierdiscus* populations, suggesting that the method eliminates complications related to the mass of different macrophyte species and macrophyte preferences and therefore allows data to be compared across studies. The Tester et al. study evaluated the performance of screens during three deployments, two in Carrie Bow Cay, Belize (May 2009 and January 2012), and one in Malaysia (May 2012). Strong relationships were reported for the two deployments in Belize (r^2 = 0.992 and 0.828 for 2009 and 2012, respectively), but the regression of cell abundances on screens versus macroalgae was not significant for Malaysia, possibly due to the large tidal ranges at the site (Tester et al. 2014). These results suggested that the 24h screens may not work in all situations. Moreover, the slopes of the regression lines for the screen- versus macrophyte-based data in Belize differed between 2009 and 2012 (0.79 and 1.09), indicating there may be interannual variability that also must be accounted for.

Parsons et al. (2017) evaluated the use of monthly deployment of screens, burlap, and PVC tiles and obtained similar results; the artificial substrates could be used to predict *Gambierdiscus* cell densities on macroalgae at some (but not all) sites, but importantly, slopes varied between sites. Additionally, the use of log-transformed data resulted in slope confidence intervals that represent two-orders of magnitude of variability in abundance estimates when converted back to untransformed data. Such large amounts of variability both between sites and within best fit (regression) analyses dictate the need for caution when relying on log-transformed data to estimate cell abundances (rather than simply establishing relationships). Based on these studies, it is evident that the implementation of artificial substrates as a standardized monitoring tool as suggested by Tester et al. (2014) will not be as easy as was hoped (Berdalet et al. 2017).

The performance of an artificial substrate must be evaluated by several criteria: 1) Does the artificial substrate track the temporal variability of *Gambierdiscus* cell densities on host macrophytes? 2) Can the cell densities on artificial substrates be translated (quantified) into cell loadings on macrophytes? 3) Do artificial substrates provide consistent results across regions, seasons, and habitats? To truly test the effectiveness and reproducibility of these (and other) methods developed to monitor *Gambierdiscus* populations, they must be evaluated by multiple researchers working in multiple regions. While previous studies have focused on "proof-of-concept" to determine if 24h screen deployments could be used as a proxy for cell densities in benthic environments, none were tested consistently over time (i.e., monthly for multiple years) as would be required in a monitoring program, particularly considering the high variability in *Gambierdiscus* cell densities observed seasonally (Bomber et al. 1988; Chinain et al. 1999; Parsons et al. 2010). Additionally, the use of alternative regression techniques (rather than linear regression on log-transformed data) might help to reduce the variability in using screen data as a proxy for cell densities in the benthos. Negative binomial distribution (NBD) regression analysis is one such technique that is applicable to data sets like those typically encountered in the study of benthic HABs (i.e., non-normal data sets that contain few high-density samples coupled with many low-density samples). Such data sets also result in situations where the variance is often greater than the mean (i.e., the data are over-dispersed; Bliss 1953). NBD regression analysis is well-suited for such data sets and does not require normality (e.g., Casas-Monroy et al. 2020). Therefore, the objectives of this study were to evaluate 1) the performance of 24h screen deployments as a representative, standardized technique to monitor *Gambierdiscus* populations on macrophyte hosts in the Florida Keys and St. Thomas (US Virgin Islands) over several years; and 2) the use of NBD regression analysis to improve the precision of the screen cell densities as a proxy for benthic *Gambierdiscus* populations in the field.

2. Methods

2.1. Site descriptions

Seventy samples were collected at four open water (exposed) sites near Long Key in the Florida Keys (FLK; Figure 1a) between April 2013 and August 2015. Forty-eight samples were collected at four open water (exposed) sites around St. Thomas (STT; Figure 1b) between July 2013 and May 2015. Two of the FLK sites, Heine Grass Bed (HGB) and Tomato Patch Hardbottom (TPH), are located in Florida Bay, whereas the other two, Long Key Hardbottom (LKH) and Tennessee Reef Lighthouse (TRL), are located on the Atlantic Ocean side of the Keys. HGB is a nearshore *Thalassia* seagrass bed in approximately 2m water depth, whereas TPH is a nearshore hardbottom site (approx. 1.5m depth) consisting of soft corals, sponges, and macroalgae. LKH is an offshore hardbottom site (approx. 5m depth) consisting of soft corals, sponges, and macroalgae, while TRL is a reef flat/crest site (approx. 7m depth) consisting of hard and soft corals, sponges, and macroalgae. Further site descriptions can be found in Parsons et al. (2017).

All St. Thomas sites are located south of the island on an inshore to offshore gradient. Coculus Rock (CRK) is located near an emergent rock reef and is composed of diverse scattered stony corals on bedrock (approx. 6-7m depth). Black Point (BP) is a nearshore fringing coral reef (approx. 7-16m depth). Flat Cay (FC) is a fringing coral reef on the leeward side of a small uninhabited island (approx. 11-16m depth). Seahorse (SH) is a deep patch reef 2km offshore of St. Thomas (approx.

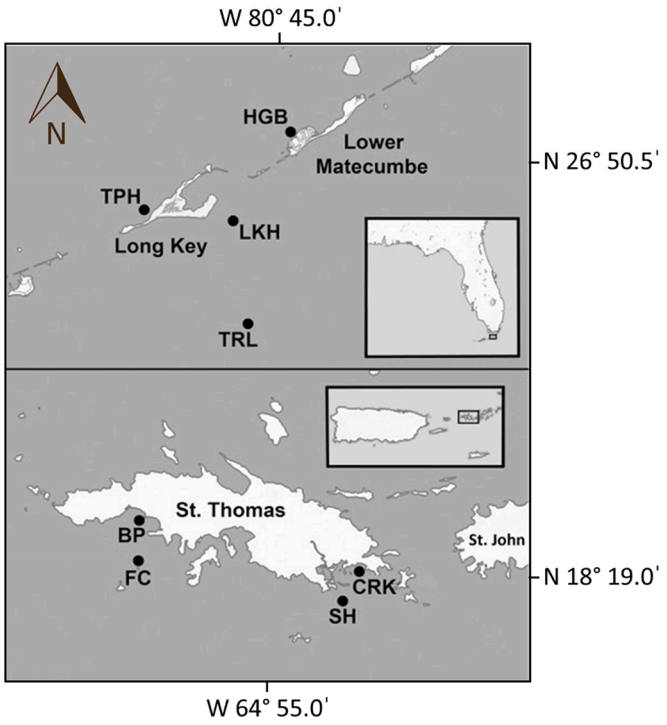

Fig. 1. Locations of the study sites in the Florida Keys and St. Thomas (four each region). HGB = Heine Grass Bed; LKH = Long Key Hardbottom; TPH = Tomato Patch Hardbottom; TRL = Tennessee Reef Lighthouse; BP = Black Point; CRK = Coculus Rock; FC = Flat Cay; and SH = Seahorse.

19-22m depth). Further site descriptions can be found in Smith et al. (2016).

2.2. Sample collection

Macrophyte samples (algae and seagrass) were chosen and collected based on their common abundance. The targeted species for the FLK sites included the brown algae *Dictyota cervicornis* (Kützing 1859) and *D. menstrualis* ((Hoyt) Schnetter, Hörning & Weber-Peukert 1987); the coralline green algae *Halimeda gracilis* (Harvey ex J. Agardh 1887) and *H. incrassata* ((J. Ellis) J.V. Lamouroux 1816); the red algae *Laurencia gemmifera* (Harvey 1853) and *L. intricata* (J.V. Lamouroux 1813); and the seagrass, *Thalassia testudinum* (K.D. Koenig 1805). STT collections focused on mixed *Dictyota* assemblages. Three triplicate samples were collected of each macrophyte, at least 10m apart from each other. This approach was validated in Parsons et al. (2017) in terms of representativeness and reproducibility. The samples were collected via SCUBA by gently placing a screw-capped polypropylene 50mL centrifuge tube over a macrophyte thallus or blade, cutting the thallus or blade at the insertion point, and capping the tube for transport back to the research vessel. The 24h screen deployments had to be incorporated into established monitoring protocols, thereby requiring some modification from the design presented in Tester et al. (2014). The FLK 24hr screen substrates consisted of fiberglass window screening mounted in wooden embroidery hoops (15 cm diameter, Joann Fabric, item #12212403), similar in nature to the monthly screen deployments reported by Parsons et al. (2017). Screen samplers deployed in St. Thomas were not mounted in hoops, but were rather suspended vertically from polyvinyl chloride (PVC) frames (0.5m in height), approximately 20 cm from the benthic substrate. Surface areas of the screens used in the FLK and STT were calculated to be 255 cm^2 and 299 cm^2, respectively.

At the FLK sites, the screen hoops were mounted onto PVC frames (46 cm per side), centered approximately 20 cm above the sediment surface as described in Parsons et al. (2017). Two screen hoops were affixed to each of two frames (approximately 40 cm apart) for a total of four replicates. Each frame was then anchored 25m apart from each other, at opposite sides of each study site. Screens at the STT sites were also suspended on PVC frames using monofilament line, and were positioned similarly. The screens were deployed each month (when weather and logistics allowed) and collected during the following day (approximately 24h, exact timing was dependent on sea state and site visit times). For collection, a 1-quart Ziploc freezer bag was carefully fitted over each screen, with care taken to not disturb the material settled on the screen. After securing the screen in the first Ziploc bag, that bag was inserted into a second bag for protection and to prevent leakage. All samples were then stowed in a mesh dive bag for transport back to the vessel.

Macrophyte and screen samples were shaken and then filtered through 200 and 20μm sieves (PVC; Nitex® mesh; 6.3 cm diameter), the Ziploc bags were refilled with 20μm-filtered ambient seawater, and shaken and filtered an additional four times to dislodge and collect the *Gambierdiscus* cells (and other loosely-attached epiphytic taxa; Richlen and Lobel 2011). At the FLK sites, one macrophyte and screen sample from each collection was shaken and filtered an additional five times through the cleaned 20 μm sieves to determine if any *Gambierdiscus* cells remained after the initial five rinse steps. These QA/QC samples were referred to as "percent recovery samples". Past studies have demonstrated that percent recovery with these methods are >95% (Parsons et al. 2017). The fourth replicate of each screen was set aside as a back-up sample as needed (e.g., leakage or substrate failure). The material collected on the 20μm sieve was then washed into a 15mL centrifuge tube using ambient filtered seawater and brought to a volume of 15mL. All tubes were then preserved with 1% glutaraldehyde (by volume) and stored on ice for transport back to the laboratory and then in a 4°C refrigerator until analyzed. STT sites were processed similarly, with total sample volumes of either 15 or 50mL. Macrophyte samples were stored back in their original 50mL centrifuge tubes with ~35mL of ambient filtered seawater and refrigerated until identified and weighed at the laboratory.

2.3. Macrophyte identification and sample size estimation

Back at the laboratory, macrophyte samples were removed from the centrifuge tubes, blotted dry, and weighed (g wet weight) on a Mettler Toledo AL204 or similar balance. The macrophytes were then identified, using keys as necessary (Littler and Littler 2000; Dawes and Mathieson 2008), and included microscopy and thallus cross-sectioning as needed.

2.4. Gambierdiscus cell enumeration

The abundance of *Gambierdiscus* cells was determined by transferring 3mL of the epiphyte or screen sample into each of three wells in a six well flat-bottomed tissue culture plate (Corning™ Costar™), stained with Calcafluor White M2R (Sigma-Aldrich Corp., St. Louis, MO) or Uvitex® (similar to calcofluor; Polysciences, Ltd., cat. #19517-10; for armored dinoflagellates), and analyzed on an Olympus IX71 (FLK) or Zeiss Axio Vert.A1 (STT) inverted microscope at 200x using a DAPI filter. Discrimination among *Gambierdiscus* species was not possible with this level of microscopy, so counts in this study are given for total *Gambierdiscus* spp. Cell counts were summed across the three wells. This approach was taken to allow for more material to be examined from each macrophyte sample (9ml total). Each well, therefore, was a subsample and each macrophyte sample was a true replicate.

The macrophyte *Gambierdiscus* data were prepared for analysis as follows. The well cell count data were summed across replicates for each macrophyte collected for each site/time sampling event (referred to hereafter as "cell counts"). The addition of 1 to the observed macrophyte cell counts ensured that the log-based regression models could accommodate cases in which this count was zero. An "offset factor" was calculated as the inverse of the product of the proportion of the samples

examined (i.e., 9mL out of the 15mL washed off of the 20μm sieve for each sample = 9/15 × 3 samples; simplified to 3/5) and the summed macrophyte wet weight (across the three replicates). Pooled samples utilized well count data summed across all macroalgal samples collected for each site/time sampling event and macrophyte wet weights summed across all macroalgal samples collected for each site/time sampling event. In summary, the *Gambierdiscus* macrophyte data were grouped as cell counts and offset factors for further analysis.

For the screen samples, the data were calculated as *Gambierdiscus* cells cm^{-2} by dividing the total cell count (+1 added as above) by the screen surface area to give density values. The screen data were then multiplied by 100 to convert values to cells 100 cm^{-2} screen to present the screen cell density values in the same orders of magnitude as cell densities on macrophytes, facilitating analysis and interpretation (Tester et al. 2014). The screen replicates were then averaged for further analysis.

2.5. Model Selection

Let Y_{jk} be the cell count on replicate macrophyte sample k at site j and let s_{jk} be the known size of the macrophyte sample. We considered three statistical models relating Y_{jk} to the cell density x_j on screens at site j: linear, log-linear, and negative binomial distribution. For economy, we will use the same notation for the parameters of these models. Under the first model, mean macrophyte cell density is assumed to depend linearly on screen density:

$$\frac{Y_{jk}}{s_{jk}} = \beta + \gamma\ x_j + \varepsilon_{jk} \tag{1}$$

where ε_{jk} is a normal error with mean 0 and variance σ^2. Under the second model, mean log macrophyte density is assumed to depend linearly on log screen density:

$$\log\frac{Y_{jk}+1}{s_{jk}} = \beta + \gamma \log x_j + \varepsilon_{jk} \tag{2}$$

Where, as before, ε_{jk} is a normal error with mean 0 and variance σ^2. This model was used by Tester et al. (2014), Yong et al. (2018) and Fernández-Zabala et al. (2019). Both of these models are normal linear regressions: for the first model, the response is $\frac{Y_{jk}}{s_{jk}}$ and the regressor is x_j while, for the second, the response is $\log\frac{Y_{jk}+1}{s_{jk}}$ and the regressor is $\log x_j$. Under the third model, the macrophyte cell count Y_{jk} is assumed to have a negative binomial distribution with mean:

$$\mu_{jk} = s_{jk}\mu_j \tag{3}$$

where:

$$\log\mu_j = \beta + \gamma \log x_j \tag{4}$$

In statistical terminology, this constitutes a generalized linear model with a negative binomial response, log link, offset $\log s_{jk}$ and regressor $\log x_j$. The negative binomial model is appropriate for over-dispersed count data. In this case, the over-dispersion arises from spatial patchiness in cells on the macrophytes. The variance of Y_{jk} is:

$$Var\ Y_{jk} = \mu_{jk} + \alpha\mu_{jk}^2 \tag{5}$$

where α is an over-dispersion parameter. As $\alpha \to 0$, the negative binomial distribution approaches the Poisson distribution.

The standard approach to selecting among these models is via Akaike's Information Criterion:

$$AIC = 2\log L_{max} - 2\ k \tag{6}$$

where $\log L_{max}$ is the maximized log likelihood and k is the number of fitted parameters, with the model with the largest value of *AIC* being selected. As the number of fitted parameters is the same for all three models, they can be compared in terms of $\log L_{max}$ alone. This is true for other model selection criteria like the Bayesian Information Criterion that essentially penalize $\log L_{max}$ by subtracting a function of k.

2.6. Data analysis

All regression analyses were conducted in the "Generalized Linear Models" routine in SPSS 25.

For each site-macrophyte pairing (e.g., HGB *Halimeda*), "cell counts" was chosen as the dependent variable. The covariate was set to "screen" (untransformed regression) or "ln(screen)" (log or NBD regression). The offset variable (which accounts for differences in macrophyte wet weight and volume of material counted; i.e., cell density) was set to "offset factor" or "ln(offset factor)" for the untransformed or log/NBD regressions, respectively. The performance of the three models was then assessed based on the log likelihood values generated for each site-macrophyte pairing. As a rough measure of unmodelled variability in algal counts, we also determined the R-squared for a regression of observed counts on their fitted values. The predictive capability of the best performing model was further examined by determining the probabilities that various fitted cell densities (i.e., estimated cell densities on the macrophytes based on the regression equation using the screen cell density data) reached threshold cell values of 1,000, 500, and 100 cells g^{-1} ww host macrophyte. The probabilities were determined using the Cdf.Normal function under the TRANSFORM tab in SPSS. Probabilities were assessed at the 90% level for four criteria: confirmed above; confirmed below; false positive; and false negative. A "confirmed above" score meant that the fitted cell densities had a 90% probability of correctly being above one of the designated threshold values. A "confirmed below" score meant that the fitted cell densities had a 90% probability of correctly being below one of the designated threshold values. A "false positive" score meant that a fitted cell density had a 90% probability of erroneously being above one of the designated threshold values, whereas a "false negative" score meant that a fitted cell density had a 90% probability of erroneously being below one of the designated threshold values.

3. Results

Screens were successfully deployed, retrieved, and counted 18 to 28 times out of the 29 months of the study for FLK, and 9 to 21 times out of the 27 months of the study for STT (Table 1). LKH and TRL had the lowest success rates at FLK (62 and 66%, respectively), primarily due to weather (rough seas) preventing retrieval after 24 hours. Similarly, the STT offshore sites (FC and SH) had lower success rates (33 and 37%, respectively), though logistical issues were the primary hurdle in these cases (i.e., time). HGB had the lowest average number of replicate screens deployed and retrieved (2.4), whereas FC and SH had the highest (3.7). Coefficients of variation ranged from 34% (TRL) to 112% (SH) for the deployments (Table 1).

An examination of the log likelihood values (Table 2) indicates that the NBD model performed best in all cases (based on the least negative values). The majority of the NBD regressions were significant ($p < 0.05$), although 38% were non-significant (8 out of 21) including all of the St. Thomas comparisons (Table 3). The LKH *Halimeda* regression had the highest r^2 value (0.74), whereas SH *Dictyota* had the worst (0.02). These relationships are distinguished in Figure 2. The average r^2 for all comparisons was 0.36, visualized in the spread of points about the 1:1 diagonal in Figure 2. When all of the data were pooled and analyzed, the r^2 was 0.16, indicating that fit did not improve when site data were combined. Overall, the fitted data tended to underestimate higher values (Figure 2), indicating a propensity of the model to produce false negatives at the high end of the spectrum.

An examination of the NBD regression slopes, intercepts and fitted cell densities revealed a large amount of variability among sites

Table 1
Summary of screen deployment metrics. The regions are the Florida Keys (FLK) or St. Thomas, USVI (STT). The FLK sites are Heine Grass Bed (HGB), Long Key HardBottom (LKH), Tomato Patch Hardbottom (TPH), and Tennessee Reef Lighthouse (TRL). The STT sites are Black Point (BP), Coculus Rock (CR), Flay Cay (FC), and Seahorse (SH). The dates signify the time frame deployments were attempted. The # of deployments indicates number of successful deployments and retrievals out of the possible number of attempts (in parentheses). The % of successful deployment and retrievals is based on these two numbers. The average number of screens retrieved and counted is provided, as is the average coefficient of variation (%CV) for each deployment.

Region	FLK				STT			
Site	HGB	LKH	TPH	TRL	BP	CR	FC	SH
Dates	04/2013 – 05/2015	06/2013 – 08/2015	04/2013 – 08/2015	06/2013 – 08/2015	07/2013 – 09/2015	07/2013 – 09/2015	01/2014 – 04/2015	01/2014 – 05/2015
# of deployments (possible)	24 (26)	18 (29)	28 (29)	19 (29)	21(27)	21(27)	9(27)	10(27)
% successful deployments	92%	62%	97%	66%	78%	78%	33%	37%
Average number of screens used	2.4	2.8	2.6	3.1	3.8	3.5	3.7	3.7
%CV	50%	46%	38%	34%	97%	112%	75%	111%

Table 2
Log likelihood values for the three regression models used on the algae and screen data. NBD = negative binomial distribution analysis.

Macrophyte Group	n	Untransformed	Log	NBD
HGB pooled	25	- 178.6	- 172.7	- 149.6
HGB *Halimeda*	25	- 167.7	- 162.4	- 134.6
HGB *Thalassia*	25	- 218.4	- 196.3	- 131.4
LKH pooled	16	- 138.1	- 74.3	- 67.4
LKH *Dictyota*	16	- 101.5	- 74.9	- 53.6
LKH *Halimeda*	16	- 57.9	- 59.9	- 47.5
LKH *Laurencia*	15	- 69.0	- 117.9	- 59.5
TPH pooled	27	- 227.3	- 227.1	- 172.2
TPH *Dictyota*	22	- 202.6	- 1502.9	- 112.3
TPH *Halimeda*	27	- 173.5	- 201.5	- 146.8
TPH *Laurencia*	27	- 190.4	- 332.1	- 149.3
TRL pooled	19	- 122.9	- 186.3	- 98.5
TRL *Dictyota*	19	- 121.1	- 182.1	- 81.8
TRL *Halimeda*	19	- 102.4	- 187.0	- 88.7
FLK pooled	87	- 669.3	- 3992.1	- 543.0
BP *Dictyota*	22	- 112.0	- 107.8	- 87.3
CR *Dictyota*	8	- 57.6	- 30.2	- 26.8
FC *Dictyota*	10	- 70.7	- 31.7	- 31.6
SH *Dictyota*	10	- 43.5	- 62.6	- 35.1
STT pooled	50	- 303.9	- 780.1	- 195.4
All pooled	137	- 1089.8	-13224.4	- 754.5

(Table 3). The slopes ranged from negative values (-0.36; FC *Dictyota*) to 0.77 (TRL *Dictyota*). The intercept data was even more variable, ranging from -0.01 (LKH *Halimeda*) to 5.97 (FC *Dictyota*). These large ranges in the slopes and intercepts (as they apply to log-transformed data) translate into greater variability in the fitted cell value data, in which screen cell densities of 3,600 cells 100 cm^{-2} (the value needed to output an average of 1,000 cells g^{-1} ww macrophyte) produced cell densities ranging from 20 (FC *Dictyota*) to 1,848 (TRL *Dictyota*). Large variability is also evident within the same macrophyte between sites (e.g., *Halimeda*; 208 to 843 cells g^{-1} ww), different macrophytes within sites (e.g., *Dictyota, Halimeda*, and *Laurencia* at LKH and TPH; ranging from 134 to 297 at LKH and 589 to 1038 cells g^{-1} ww at TPH), as well as pooled macrophyte data between sites (112 to 958 cells g^{-1} ww).

The relatively low r^2 values (Table 3) coupled with the associated high variability resulted in relatively poor predictive capability in the NBD model: for example, only 40% of the predicted values had a 90% or greater probability of meeting the actual algal cell density (i.e., "confirmed"). While the NBD model performed well in terms of accurately confirming that fitted values were below the higher thresholds (1000 and 500; confirmed below), the model performance decreased substantially in the lower thresholds. The model did not produce many false positive results (only 11), but did not accurately categorized fitted values above each threshold ("confirmed above"), resulting in many false negative results. Overall, the model performed better at high threshold values rather than low threshold values.

Table 3
Regression parameters for the NBD models for the site-macrophyte comparisons and the resultant fitted cell densities based on 3,600 cells 100 cm^{-2} screen (the value needed to give an average of 1,000 cells g^{-1} ww for the fitted data).

Site	Macrophyte	p-value	r^2 value	slope	intercept	over-dispersion parameter	fitted density
HGB	pooled	<0.0005	0.64	0.60	1.97	0.56	958
HGB	*Halimeda*	<0.0005	0.60	0.64	1.46	0.55	843
HGB	*Thalassia*	<0.0005	0.58	0.48	3.41	0.70	1550
LKH	pooled	<0.0005	0.62	0.47	1.22	0.20	154
LKH	*Dictyota*	0.001	0.55	0.41	2.33	0.35	297
LKH	*Halimeda*	<0.0005	0.74	0.65	-0.01	0.09	208
LKH	*Laurencia*	0.085	0.21	0.35	2.01	0.59	134
TPH	pooled	<0.0005	0.73	0.74	0.76	0.72	935
TPH	*Dictyota*	0.372	0.04	0.39	3.76	0.82	1038
TPH	*Halimeda*	<0.0005	0.73	0.66	1.00	0.61	589
TPH	*Laurencia*	0.001	0.39	0.62	1.68	0.95	875
TRL	pooled	0.030	0.25	0.52	1.74	0.57	413
TRL	*Dictyota*	0.012	0.32	0.77	1.23	0.62	1848
TRL	*Halimeda*	0.071	0.18	0.5	1.70	0.62	330
FLK	pooled	<0.0005	0.28	0.17	4.25	1.47	275
BP	*Dictyota*	0.137	0.11	0.17	3.08	0.36	85
CR	*Dictyota*	0.558	0.06	0.15	3.79	0.37	153
FC	*Dictyota*	0.063	0.37	-0.36	5.97	0.08	20
SH	*Dictyota*	0.708	0.02	0.07	2.76	0.72	29
STT	pooled	0.060	0.07	0.18	3.28	0.58	112
All	pooled	<0.0005	0.16	0.16	4.02	1.30	212

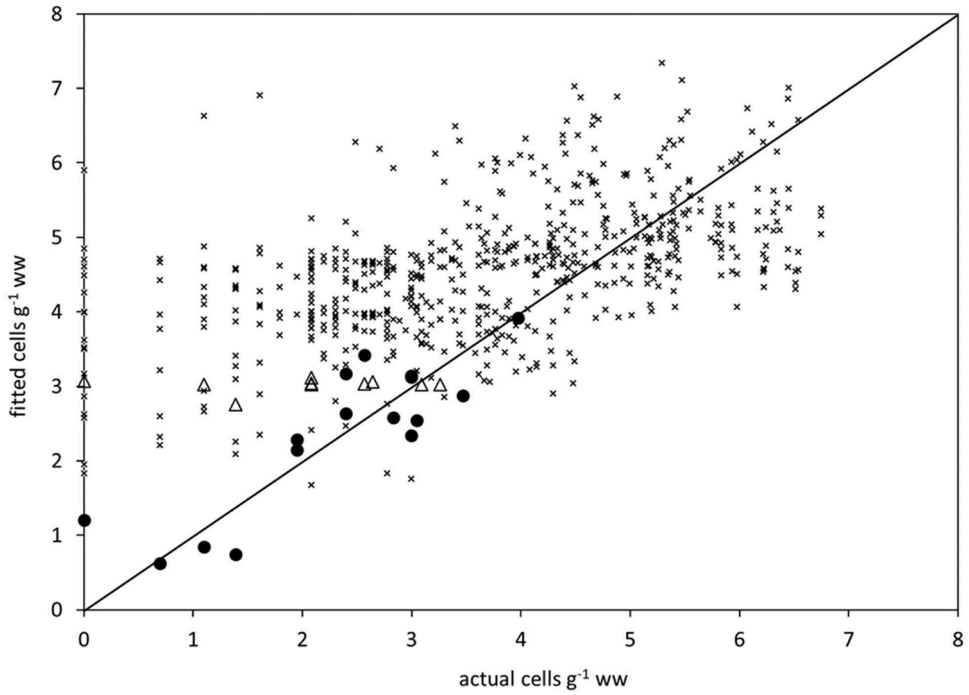

Fig. 2. Fitted versus actual cell densities (cells g^{-1} ww; ln-transformed) for all samples analyzed in this study. The data exhibiting the best r^2 values (LKH *Halimeda*) are shown as black circles. The data exhibiting the worst r^2 values (SH *Dictyota*) are shown as triangles. A 1:1 line is also fitted to the graph.

4. Discussion

A major goal in ciguatera research is the development of a standardized sampling method for *Gambierdiscus* cell abundance (Berdalet et al. 2017). Artificial substrates in particular have been explored as a potential alternative that eliminates concerns regarding potential host preferences and weight-to-surface area variability inherent among the different macroalgae morphologies. Tester et al. (2014) proposed the use of 24h deployments of window screens as that standard, for broad adoption by the scientific community. The results of that initial study, however, demonstrated that 24h screen performance was not consistent across space and time, significantly hindering its use as a standardized method. For example, while *Gambierdiscus* cell densities on the 24h screens were significantly correlated with cells densities on macroalgae in Belize, they were not correlated in Malaysian waters. A second study conducted in Malaysian waters again demonstrated that 24h screens did not serve as a representative proxy for macroalgae, or benthic microhabitats in general (Yong et al. 2018). Similarly, screens worked for many (but not all) of the sites in the Canary Islands (Fernández-Zabala et al. 2019). We report similar results in this study: *Gambierdiscus* cell densities on screens were correlated with those on macroalgae in most comparisons, but correlations were poor for the St. Thomas sites. Part of this may have been due to different designs; i.e., screens were mounted in hoops at the FLK sites and were bare at the STT sites (similar to Tester et al. 2014). If the design played a factor, however, then the Tester et al. (2014) design used at the STT sites performed poorly, whereas the hoop mounted screens were better suited for the FLK sites. Identification of factors contributing to the performance of screens, including the characterization of habitats/systems in which they can be used, is clearly needed before widespread implementation of this method.

Ecologically, wave energy may partially explain why screens work in some places and not others (e.g., Fernández-Zabala et al. 2019). In our study, the STT sites experience higher wave energies than two or three of the FLK sites (the two bay sites – HGB and TPH; and the nearshore site – LKH). *Gambierdiscus* cells have been observed in the water column (reviewed in Parsons et al. 2012; analyzed in Stanca and Parsons 2017), and would need to swim or be advected from the benthos to the 24h screen for colonization and settlement. Nakahara et al. (1996) reported that turbulence caused *Gambierdiscus* cells to stop swimming and attach to the substrate via mucus. Under these circumstances, *Gambierdiscus* cells would firmly attach to substrate, thereby preventing advection to nearby collecting screens in the water column. Such a scenario may explain the difference in the Belizean data presented in Tester et al. (2014) for 2009 (May) versus 2012 (January). It is possible, therefore, that screens may be less effective at sites (or during seasons) experiencing higher wave energy. That being said, site selection should not be based (solely) on wave energy, but rather on locations that are extensively fished and/or are known hot spots (e.g., south shore of St. Thomas; Loeffler et al. 2018). If artificial substrates cannot adequately monitor such locations, then other methods will need to be developed and utilized.

Another possibility is that some *Gambierdiscus* species may be more active swimmers than others, and therefore more likely to be advected to a screen (or *vice versa*). Rains and Parsons (2015) demonstrated that there is variability among *Gambierdiscus* species in how they interacted with different macroalgal hosts. In culture, differences in the degree to which various *Gambierdiscus* isolates/species produce mucous and adhere to the sides and bottom of the culturing vessel are also evident (pers. observations). The species composition of *Gambierdiscus* varies among sites and seasons in both St. Thomas and the Florida Keys (Richlen, unpub. data), therefore it is likely that the fraction of cells that are swimming and advecting versus those that remain firmly attached to the substrate will also vary. Such changes in species composition will create variability in slopes between *Gambierdiscus* cells on screens versus macroalgae as this fraction (ratio) changes. As molecular techniques are implemented in more field studies to explore community composition dynamics (e.g., Pitz et al. 2021), this second hypothesis can be easily tested by comparing species compositions between screens and macroalgae across seasons and sites. Similarly, variability in physical-chemical parameters (e.g., nutrients, light and temperature) may influence *Gambierdiscus* behavior (e.g., retreating to the underside of macroalgal blades in high light conditions; Villareal and Morton 2002) and species composition at a given site. Lastly, variability within the benthic microenvironment may play a significant role, in that *Gambierdiscus* cell

densities may be higher on non-targeted substrates (e.g., turf algae; Yong et al. 2018), thereby influencing the distribution of cells on targeted substrates (e.g., *Dictyota, Halimeda, Laurencia*, and *Thalassia* in this study). A similar substrate effect may occur for other benthic dinoflagellates (e.g., *Ostreopsis* and *Prorocentrum*) that are commonly found on sand surfaces and in the water column (Fernández-Zabala et al. 2019).

When screens do appear to track *Gambierdiscus* cell densities on macrophytes, however, the results vary spatially and temporally. For example, the regression equations reported in Tester et al. (2014) differed between years for the Belize samples, exemplified through the example where 1,000 *Gambierdiscus* cells g^{-1} wet weight algae (the lower limit of the threshold proposed by Litaker et al. (2010) for increased ciguatera risk) equated to 5,070 cells 100 cm^{-2} screen in 2009 versus 8,203 cells 100 cm^{-2} screen in 2012, 62% higher. These results suggest that screens may have to be recalibrated year to year at individual sites to properly monitor for the 1,000 cell threshold. In addition to such temporal variability concerns, there is well-documented site to site variability, depicted by the different slopes of regression equations across sites in the Canary Islands (Fernández-Zabala et al. 2019) and in this study (Table 3). Screens therefore will likely need to be calibrated site to site as well.

The inconsistency of 24h screens across sites (and time) merits further discussion. Are there reasons for their variable performance? From a statistical standpoint, the variability seen in this study could be due in part to the lower number of replicate screens used (generally three) versus previous studies (e.g., seven in Fernández-Zabala et al. 2019). Our results, however, were consistent with previous studies mentioned above. For example, Parsons et al. (2017) previously demonstrated that three replicates were adequate to meet the N_{100} criteria (CV <100%) first proposed by Tester et al. (2014) for the monthly-deployed artificial substrates and macrophytes in that study. In the current study, six out of eight of the sites had average coefficients of variation <100% (Table 1). The two sites that had higher coefficients (Coculus Rock and Seahorse in St. Thomas; 111% and 112%) may have benefitted with more replicates, but the r^2 values of the regressions were extremely low (0.06 and 0.02, respectively). The use of more replicates would not have changed these outcomes. Several researchers have stressed the need to validate that a 24h deployment duration is adequate for cell densities to reach equilibrium on the screens (Tester et al. (2014) and Fernández-Zabala et al. (2019)). Our preliminary work (eight separate deployments over eight months) at HGB (Florida Keys) demonstrated that screens became saturated within 18 hours in all cases (unpubl. data). Therefore, we are confident that our results are valid and representative.

The establishment of a significant relationship between screen-based cell densities and macrophyte-based densities is not the only factor requiring testing and scrutiny when evaluating the applicability of 24h screens as a monitoring tool for *Gambierdiscus* populations. The strength of the relationship (i.e., goodness of fit) must also be addressed. The high degree of variability exhibited in *Gambierdiscus* cell densities on macrophyte hosts and screens resulted in non-normal distributions, requiring the use of transformed data or non-parametric analyses. The use of untransformed and transformed data each provided advantages and disadvantages (Parsons et al. 2017). Log-transformed data were utilized in the Tester et al. (2014) study and subsequent studies, thereby representing a potential standardized approach to data analysis for this method. We found that negative binomial distribution regression models performed better than untransformed or log-transformed regression models (Table 2), but the performance was still inadequate. Not only were screens ineffective at some sites (St. Thomas) and produced a high degree of site to site variability, but there was evidence that individual screen deployments (i.e., screens collected and analyzed from each sampling trip) must also be assessed on a case by case basis. Even when models were statistically significant (Table 3), they ran a risk of creating both false positive and false negative results that could severely hinder the use of screens to monitor *Gambierdiscus* in the benthos (Table 4). For example, Litaker et al. (2010) proposed a threshold of 1,000 cells g^{-1} wet weight algae, above which cases of ciguatera poisoning were thought to be more likely. When a screen-based monitoring program (calibrated for that current year and particular site) produces estimated cell densities, there is a chance that the estimated value could be erroneous; it may be too low (producing a false negative) or too high (producing a false positive). The performance of such monitoring systems must be assessed prior to their implementation (e.g., Stumpf et al. 2009).

An important caveat that must also be considered is that high cell densities do not necessarily translate into high toxin content in the *Gambierdiscus* community at a site. Researchers have traditionally linked ciguatera outbreaks to epibenthic "blooms" of *Gambierdiscus* (e.g., Withers 1983; Bagnis et al. 1990), where higher numbers of cells were thought to lead to higher amounts of ciguatoxin moving up into the food web and subsequently leading to ciguatera poisoning in people who consumed toxic fish. When toxin level per cell (i.e., toxin content) was determined, however, it became clear that some cells produced more toxin than others. For example, Holmes et al. (1991) reported that only two of thirteen isolates of *Gambierdiscus* produced ciguatoxin, and suggested that the mere presence of *Gambierdiscus* did not mean that a ciguatera outbreak was imminent, but that a toxic strain had to be present. This conclusion is corroborated by Chinain et al. (1999), who reported that although *Gambierdiscus* cell concentrations followed a quasi-seasonal cycle (peaking at the beginning and end of the hot season) in field studies conducted in French Polynesia, total toxicity of the cells did not follow the same cycle, suggesting that cell abundance and toxin production were not synchronized. Lastly, Robertson et al. (2018) reported that the ciguatoxicity of *Gambierdiscus* varies at least 2000 × among species and strains collected and analyzed from St. Thomas and the Florida Keys. Therefore, as is the case with other HAB species, the simple monitoring of *Gambierdiscus* abundance will not allow one to forecast a ciguatera event. Steps have been taken by some research groups to overcome these discrepancies by developing techniques to specifically quantify the most toxigenic *Gambierdiscus* taxa (e.g., FISH probes; Pitz et al. 2021) and to monitor the dissolved fraction of ciguatoxin in the water column (e.g., SPATTs, Roue et al. 2020). Continued refinement of such techniques will vastly improve our capabilities to monitor *Gambierdiscus* populations and their toxin production, thereby providing the means to forecast and prepare for possible ciguatera outbreaks.

Probably the most important factor that must be considered is the trophic transfer of ciguatoxins (or precursors). The initial transfer of these compounds into demersal (reef) food webs is via herbivory on host macroalgae harboring *Gambierdiscus* cells (Yasumoto et al. 1977). The palatability of host macroalgae plays an important role in this transfer; i.e., all macroalgae species are not equally consumed by herbivores (Cruz-Rivera and Villareal 2006). Cruz-Rivera and Villareal (2006) identify the palatable hosts as potential vectors for ciguatera, whereas

Table 4
Number of cases (out of 622 total) where fitted cell density estimates (NBD) were classified as confirmed above, confirmed below, false positive, or false negative versus target (threshold) cell density values on algae. A probability of 90% was used as the cut-off value to determine if a fitted value reached each threshold. Please refer to the text on more detailed definitions of the scoring criteria.

Threshold (cells g ww^{-1})	Confirmed above	Confirmed below	False Positive	False Negative
1000	0% (0)	100% (622)	-	-
500	0% (0)	94.9% (590)	0% (0)	5.1% (32)
100	0% (0)	71.4% (444)	0% (0)	28.6% (178)
50	0.2% (1)	55.5% (345)	0% (0)	44.3% (276)
10	7.1% (44)	16.7% (104)	1.8% (11)	74.4% (463)

non-palatable hosts act as refuges. What if non-palatable "refuge" macrophytes are the source of the majority of *Gambierdiscus* cells that settle on the screens? How does the palatability of the various macrophytes located in the vicinity of screen deployments factor into the risk assessment?

A critical oversight to date has been the lack of focus on turf algae, particularly in artificial substrate (screen) studies. Turf algae have long been considered a primary vector of toxin transfer (Randall 1958; Helfrich and Banner 1968), and should be the focus of any monitoring program. For example, Kopp et al. (2010) examined grazing by herbivorous fishes at reef sites around Guadeloupe, and found that turf algae were preferentially grazed upon, whereas brown algae were avoided. Additionally, efforts need to be pursued and expanded to quantify ciguatoxin concentrations in macrophyte material to better document the (potential for) trophic transfer of the toxins into the food web. The usefulness of screen deployments to monitor and track these processes has not yet been demonstrated.

As discussed earlier, *Gambierdiscus* species display different host preferences or behaviors (swimming versus attachment; Rains and Parsons 2015). Coupling host preferences of different *Gambierdiscus* species in the context of host palatability and *Gambierdiscus* toxicity, Rains and Parsons (2015) concluded that the presence of *G. belizeanus* on *Polysiphonia* and *Dictyota* were the most likely candidate vectors for the trophic transfer of ciguatoxin into the food web based on the *Gambierdiscus* – host algae combinations tested. Other *Gambierdiscus* species are more toxic (e.g., *G. excentricus* and *G. polynesiensis*), and other algae (e.g., turf species) are likely more palatable hosts, but this study demonstrated that it is the combination of all three factors that ultimately determines potential fish toxicity. When particularly toxigenic cells colonize a preferred food source of grazers, the transfer of ciguatoxins into the food web will likely be amplified and accelerated. A neutral (artificial) substrate, therefore, may not adequately capture either the host preference or palatability that are likely significant influential factors in the initial phase of toxin incorporation in the food web. Rather than monitoring *Gambierdiscus* populations in an isolated manner that eliminates herbivory from the equation, a better, more applicable approach would be to develop a simple, standardized sampling method that directly relates to fish toxicity (e.g., CTX-eq. g^{-1} wet wt algae or cm^{-2} (m^{-2}) substrate). Such a method would require vetting, however, including the identification of indicator fish for toxin validation.

While the use of artificial substrates held great promise in the standardization of protocols to monitor *Gambierdiscus* populations around the globe, their performance has not been adequately tested, and results from existing studies have been inconsistent and variable both temporally and spatially (Tester et al. 2014, Parsons et al. 2017, Yong et al. 2018, Fernández-Zabala et al. 2019). Careful consideration must be given when implementing 24h screens into a monitoring program; are resources available for many replicate screens and for the doubling of site visits to deploy and retrieve screens? Will screens need to be calibrated every year and for every site?

The 24h screens can provide information that is important ecologically, as long as the user is careful what questions are being addressed. For example, the screens are a good tool to study advective transport of *Gambierdiscus* spp. and other epiphytic dinoflagellates within a site, or between sites. They also could be used to study emigration and immigration dynamics through time, such as on a diel cycle, over the course of seasons, or before, during and after storms. We believe that more work is needed, however, before screen deployments can be implemented into risk assessment protocols for ciguatera.

While great strides have been made in ciguatera research, there are still many unknowns that must be addressed and methods that need to be evaluated, modified, or developed. Efforts to enumerate toxic cells and to quantify the transfer of toxins through the food web are particularly challenging given the benthic habitat and epiphytic lifestyle of *Gambierdiscus* spp., as well as significant differences in toxicity among species comprising the communities sampled. The search for standardized approaches should continue, but careful evaluation across sites and time is needed before broad adoption is recommended.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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