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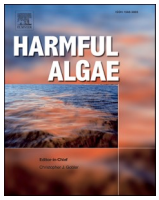

Using artificial substrates to quantify *Gambierdiscus* and other toxic benthic dinoflagellates for monitoring purposes

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ABSTRACT

Collecting methods generally used to determine cell abundances of toxic benthic dinoflagellates (BHAB) use cells dislodged from either macrophytes or artificial substrates. This article compares the advantages of the macrophyte and artificial substrate methods and discusses which method is more appropriate for use in monitoring programs that focus on toxic BHAB species identification and quantification. The concept of benthic dinoflagellate "*preference*" for specific macrophytes was also reviewed. Examination of data from 75 field studies showed macrophytes with higher surface area per unit biomass harbored higher concentrations of *Gambierdiscus* cells. There was no definitive evidence that cells were actively selecting one macrophyte over another. This observation supports the use of artificial substrates (AS) as a means of assessing cell abundances in complex habitats because cell counts are normalized to a standardized surface area, not macrophyte biomass. The artificial substrate method represents the most robust approach, currently available, for collecting toxic, benthic dinoflagellates for a cell-based early warning system.

1. Introduction

Ciguatera poisoning (CP) is a long-neglected malady affecting tropical regions of the Pacific and Indian Oceans, the Caribbean Sea and more recently, Macaronesia. It is caused by the bioaccumulation of ciguatoxins (CTXs) produced by benthic dinoflagellates in the genera *Gambierdiscus* and *Fukuyoa* (referred to hereafter only as *Gambierdiscus*) in marine food webs. In light of increasing ocean temperatures and sea level rise, CP is of special concern regarding food security for small, tropical, island nations. The Pacific Nations participating in the 32nd Session of the Committee on Fisheries, 2016 specifically raised CP "… *as an issue that increasingly affects the tropical and subtropical regions of the Pacific Ocean, Indian Ocean, and Caribbean Sea, between the latitudes 35°N and 35°S. Indeed, it was noted that due to climate change the frequency of storms and hurricanes increases as well as the sea surface temperature (SST) which impacts on the distribution and proliferation of the ciguatera-toxins (CTX) and makes the occurrence of CFP less predictable (Food and Agriculture Organization of the United Nations* FAO 2016)." The following year, CP was a featured agenda item during the 11th Session of the Codex Committee on Contaminants in Food (CCCF 2021).

After hearing testimony, the 2017 Codex Committee requested scientific information and in late 2018 a joint Food and Agriculture Organization–World Health Organization (FAO-WHO, UNESCO) meeting of experts was held to provide advice on the development of risk management options for CP. As a consequence of the Report of the Expert Meeting on Ciguatera Poisoning (FAO and WHO, 2020), a task team was asked to develop implementation plans for Early Warning Systems (EWSs) for Harmful Algal Blooms (HABs) including benthic species (Technical Guidance for the Implementation of Early Warning Systems for Harmful Algal Blooms, FAO, International Oceanographic

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Commission, International Atomic Energy Agency, in press). After a decade of productive research on the genus *Gambierdiscus*, which revised its taxonomy, augmented our understanding of its global distribution and introduced molecular methods for cell detection and enumeration, we are on the threshold of standardizing and codifying methodologies for a cell-based early warning system.

Key decision points for standardizing protocols for monitoring *Gambierdiscus* are how, when and where to sample. Unlike planktonic species, there is no standardized, or even agreed upon, quantitative method for sampling benthic dinoflagellates. Traditionally, macrophyte hosts have been collected and cell abundances normalized to grams wet weight of the "*host*" macrophyte. While this method is widely used for preliminary assessments of habitats for presence-absence, the macrophyte collecting method is inadequate for quantification of BHABs. There are myriad arguments for why the macrophyte collection technique is unacceptable, not the least of which is that normalization based on macrophyte biomass is biased due to the different surface area to volume ratios of macrophyte species. Artificial substrates (AS) are proposed as a better method because cell abundances are normalized to a precisely quantifiable surface area.

This review focuses on the literature regarding sampling toxic *Gambierdiscus* species, but much of the information presented will be applicable to other benthic genera including *Amphidinium, Coolia, Ostreopsis, Fukuyoa, Prorocentrum* and *Vulcanodinium* (Tester et al., 2014; Tester and Kibler, 2018; Bravo et al., 2020). Emphasis is placed on the advantages and disadvantages of the two most common sampling methods used in structurally complex habitats, the expected variability of each method and the replicability needed to obtain statistically robust cell abundance estimates. Our goal is to correct errors in attributions, dispel misinterpretations and dismiss nonproductive models of the relationship between macrophyte and AS collecting methods. For considerations of other sampling approaches, not addressed in this review, see Hoppenrath et al. (2004), Moreira and Tester (2016), Mangialajo et al. (2017) and Tester and Kibler (2018).

2. Sampling methodology and challenges

A brief overview of the challenges associated with sampling benthic dinoflagellates and a historical review of the development of the macroalgal and artificial screen sampling methods follows. In addition, the two methods are compared with regard to estimating cell densities.

2.1. *Gambierdiscus and other toxic benthic dinoflagellates are found in complex habitats and exhibit patchy distributions that complicate sampling*

Dinoflagellates are often abundant members of shallow water, marine, benthic habitats (Hoppenrath et al., 2014; Durán-Riveroll et al., 2019). A subset of these benthic species, especially members of the genera *Gambierdiscus* and *Ostreopsis*, can produce toxins or mucilage or both, that disrupt normal ecosystem functions and adversely impact human and animal health (Berdalet et al., 2017; Litaker et al., 2017; Larsson et al., 2018). Because of these adverse impacts, there is a need to quantify species-specific changes in cell abundances and distributions for monitoring purposes.

Unlike pelagic phytoplankton where monitoring can be achieved by collecting samples from a relatively uniform medium (either by integrating the water column or at discrete depths), benthic dinoflagellates are found adhered to many substrate types including algal turf, macroalgae, rocks, coral rubble, sand or seagrasses (Yong et al., 2018) and artificial structures such as concrete seawalls and pilings (Meroni et al., 2018; Villareal et al., 2007). Further, *Gambierdiscus* abundances can vary by an order of magnitude between adjacent macrophytes only 5–10 cm apart (Taylor and Gustavson, 1986). Complex surface areas, patchy distributions of macrophytes and variable *Gambierdiscus* abundances all confound sampling strategies for quantifying benthic dinoflagellates.

2.2. *History of the macrophyte and artificial substrate sampling methods*

It was recognized early that *Gambierdiscus* and other benthic genera were commonly found on macrophytes and exhibited highly variable distributions. Ballantine et al. (1985) found *Gambierdiscus* cells on *Dictyota* collected within a 3 m radius varied by more than an order of magnitude. Lobel et al. (1988) were the first to quantify this variability and demonstrate the number of replicates required to estimate *Gambierdiscus* abundance. They recognized the "need" for a standardized, statistically rigorous methodology for examining the distribution and *abundance* of CP causing dinoflagellates among sampling locations and over time. Briefly, macrophytes were collected and shaken vigorously in a container with seawater to dislodge benthic cells adhering to the surface of the macrophyte. The samples were sieved to remove larger particles and *Gambierdiscus* or other benthic species were concentrated and counted. The wet weight of the host macrophyte was determined and used to normalize cell abundances reported as cells per g^{-1} wet weight (mass) of macrophyte.

Lobel et al. (1988) also recognized how the large variation in biomass to surface area among macrophytes would complicate estimating *Gambierdiscus* cell densities. For example, if the same weight of macroalgae from different species with different weight to surface area ratios was sampled, and they yielded the identical number of BHAB cells, the resulting density estimates would be very different depending on whether they were normalized using wet weight or surface area. They focused their quantitative study on only two macrophyte genera to assess *Gambierdiscus* cell densities, *Dictyota* (Ochrophyta) and *Galaxaura* (Rhodophyta). They estimated the surface area of *Dictyota* collected in the field was 105 ± 31 cm^2 g^{-1} ($n = 13$) compared to 31 ± 8 cm^2 g^{-1} ($n = 4$) for *Galaxaura*. Next, estimated cell densities normalized to both g wet weight macrophyte and surface area were calculated. The resulting normalized cell densities on *Dictyota* averaged 24 ± 14 cells g^{-1} wet weight algae or 23 cells cm^{-2} compared to 6 ± 10 cells g^{-1} wet weight algae or 19 cells cm^{-2} for *Galaxaura*. The maximum density never exceeded 56 cells g^{-1} wet weight algae for *Dictyota* or 30 cells g^{-1} wet weight algae for *Galaxaura*. At these low densities, the calculated standard error of the mean was never less than 200% based on the number of individual macrophytes sampled. After conducting a power analysis that included replicate numbers ranging from 3 to 20 macrophytes per site, Lobel et al. (1988) recommended a *minimum* of 10 replicates be taken at each sampling site to have a statistically acceptable estimate of *Gambierdiscus* abundance when cell abundances were low. As a result of these findings, they suggested comparable abundances from different sites could only be obtained by sampling macrophyte species from each site for which the surface to volume ratios were known. They stated that *"Until surface area to mass relationships were determined for other important macroalgae, [Gambierdiscus] abundance data cannot be interpreted either in terms of substrate preference or geographic distribution patterns. Only data for the same host species would be comparable, and then only if the number of replicate samples was sufficient."*

The Lobel et al. (1988) study results, coupled with *Dictyota's* wide-ranging distribution, accounts for the emphasis on sampling this species as means of obtaining comparable *Gambierdiscus* abundances from different sites (Ballantine et al., 1988; Irola-Sansores et al., 2018; Liefer et al., 2021). Using cell density estimates from *Dictyota* to represent the overall relative concentration of *Gambierdiscus*, however, requires two assumptions. First, *Gambierdiscus* cell abundance estimates from *Dictyota* must be representative of the overall distribution of benthic species. Second, the distribution of *Dictyota* must be sufficiently uniform in time and space that an adequate number of samples can be collected consistently in all habitats. Neither of these assumptions can be met in most cases. Despite the caveats raised by Lobel et al. (1988), sampling macrophytes and normalizing cell densities on a biomass basis has remained the primary method for estimating the abundance of most BHABs. This method was not challenged or improved upon until new *Gambierdiscus* and other BHAB species descriptions began increasing

after 2009 (Litaker et al., 2009). Some species were shown to vary in toxicity and to occur in multiple locations, some of which did not support *Dictyota* or where *Dictyota* phenology varied (Tester et al., 2014; Bosinoir et al., 2018; Fernández-Zabala et al., 2022). This situation raised the question as to whether it was possible to develop an alternative, standardized, easy to use sampling method capable of providing comparable BHAB cell estimates across sampling sites and times. Ideally, the method developed could be incorporated into ongoing monitoring programs to support early warning systems like the one being developed for FAO (in press).

2.3. Artificial substrate sampling method

The AS sampling method for BHABs proposed by Tester et al. (2014) was developed in response to the demonstrated need for a standardized, statistically robust sampling method. The method itself was inspired by observations made while sampling benthic dinoflagellates in habitats near the Smithsonian field station at Carrie Bow Cay, Belize. Faust (2009) made the initial observation that benthic dinoflagellates could be easily collected by hanging a frayed plastic rope in the water column overnight and then rinsing it to collect BHAB cells. This observation and an understanding that species like *Gambierdiscus* are more epibenthic, and less sessile than previously reported (Ballantine et al., 1988; Nakahara et al., 1996), led to the following questions. How motile are benthic species? How readily do they colonize available benthic surfaces? If they move and resettle frequently, would this provide a means of averaging their overall abundance in structurally complex habitats? Given the success of the rope collection technique, benthic dinoflagellates were hypothesized to be sufficiently motile to rapidly colonize free surfaces in proportion to their overall abundance in the surrounding habitats. This behavior would allow AS to be used to quantitatively estimate benthic dinoflagellate abundances based on surface area rather than macrophyte biomass (see Moreira and Tester 2016; Tester and Kibler, 2018, Jauzein et al., 2018; Vassalli et al., 2018).

To test this hypothesis, fiberglass window screens were deployed as artificial substrates to collect benthic dinoflagellate samples (e.g., Tester et al., 2014; Jauzein et al., 2018; Fernández-Zabala et al., 2019). This substrate was selected because fiberglass screens are lightweight, easy to deploy, inert, inexpensive, available worldwide and have precisely quantifiable surface areas (Fig. 1). They can be discarded after use or cleaned to eliminate sample contamination. Briefly, rectangles of precisely measured window screen material were anchored a few cm above the bottom with a small float added on a short line at the top to keep the screens suspended.

After 24 h, the screens were carefully collected in wide mouth jars, sealed and returned to the laboratory for processing. This included shaking the jar to dislodge the cells from the screene and sieving to remove any larger debris, before concentrating and preserving the cells using a neutral Lugol's solution. At the same time, macrophytes in the vicinity of the screens were collected and processed using the traditional macrophyte sampling method described above.

2.4. Comparison of data from artificial substrate studies

Preliminary studies completed in Belize in 2010–2012 revealed similar log linear correlations among the *Gambierdiscus, Ostreopsis* and *Prorocentrum* cell concentrations estimated as cells g^{-1} wet weight obtained from macroalgae and cells 100 cm^{-2} obtained from nearby screen samples. A determination of how quickly the screens were colonized was accomplished by deploying a set of window screens and subsampling at 6, 12, 18, 24, 36 and 48 h intervals (Tester et al., 2014). The screens recruited cells for approximately 24 h at which time cell densities remained nearly constant. The 24 h loading period required for equilibrium cell densities was subsequently confirmed for *Gambierdiscus, Prorocentrum* (Fernández-Zabala et al., 2019) and *Ostreopsis* (Jauzein et al., 2018).

In 2012 an international workshop was held in Malaysia to teach the AS method and to carry out a proof-of-concept field sampling exercise comparing cell counts of *Gambierdiscus, Ostreopsis* and *Prorocentrum* from macroalgae and AS sampling devices placed in the same habitats. The placement of the screens within algal beds was determined using a randomized design. The first outcome of the workshop was the publication by Tan et al. (2013). They successfully used the AS method to estimate *Amphidinium, Coolia*, *Gambierdiscus, Ostreopsis* and *Prorocentrum* species densities along a fringing reef found off Sampadi Island, Sarawak, Malaysia. Tester et al. (2014) published the comparison of the results from the Belize and Malaysia workshop studies. The results showed a strong correlation between cell counts obtained from macroalgae and those from deployed screens. Referencing Lobel et al. (1988), a power analysis was completed to determine the number of samples needed to obtain reasonable estimates of cell abundances. At mean densities of around 100 cells cm^{-2}, 6–7 replicates were needed to reliably reduce the coefficient of variation (CV) to $\leq$100%. These results were consistent with those of Lobel et al. (1988) who estimated at least 10 macrophyte samples would be needed to achieve acceptable estimates of cell abundances at mean cell concentrations of 24 cells cm^{-2}

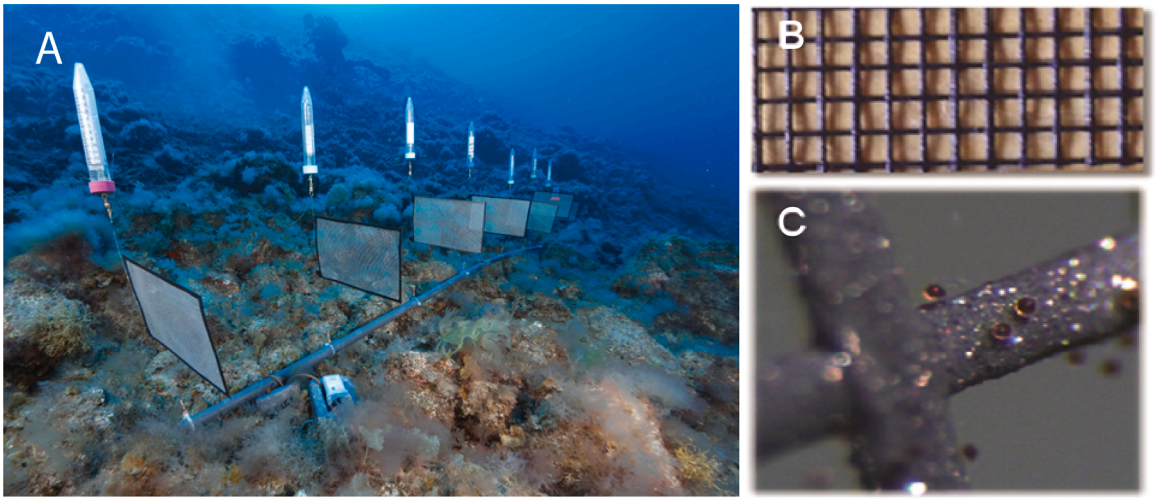

Fig. 1. Artificial substrate (window screen) sampling method. A) Sampling device with measured pieces of window screen anchored on a weighted rod with test tube floats (Fernández-Zabala et al., 2019), B) Magnified window screen mesh showing its three-dimensional structure which must be considered when estimating surface area available for colonization of cells. C) *Gambierdiscus* cells colonizing screens (NOAA).

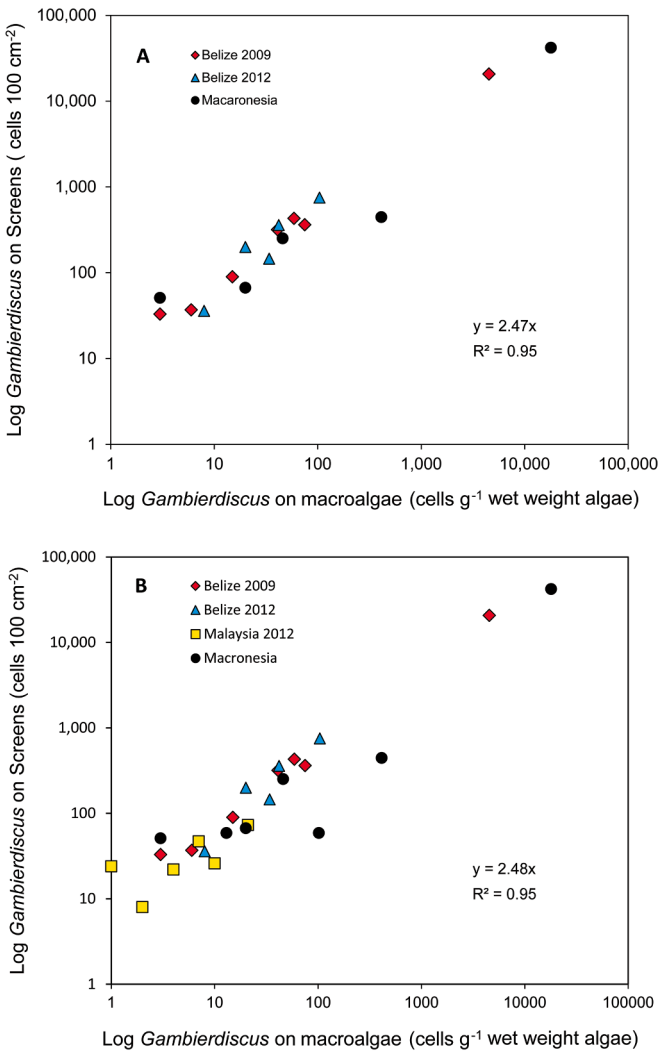

Fig. 2. The combined data of mean concentration of cells on macroalgae and nearby artificial sampling devices from Tester et al. (2014) and Fernández--Zabala et al. (2019). The number of replicates for Belize 2009 ranged from n = 5–9; n = 3 for Belize in 2012, n = 5–6, for Malaysia 2012, and n = 3–7 replicates for Macaronesia. A) Subset of the data where 5–9 replicates were used to calculate the mean cell concentrations. B) Full data set where replicate numbers ranged from 3 to 9.

(Fig. 2).

The AS method has also been successfully used to sample *Ostreopsis*. Cell densities were generally high and the heavy mucus from *Ostreopsis* necessitated the addition of a frame to support the mesh window screen. This optimization of the original design prevents screens from folding back on themselves either in high turbulence environments (Fernández-Zabala et al., 2019) or because of the weight of mucous producing cells like *Ostreopsis* on the screens (Jauzein et al., 2016,2018). This modification is recommended for incorporation into standard protocols for monitoring BHABs.

Fernández-Zabala et al. (2019) noted that, in most cases, variability of cell abundances of epiphytic dinoflagellates was lower on AS than on macroalgae in the Macaronesia region. For species whose abundances are typically low, such as *Gambierdiscus* (<100 cells g^{-1} wet weight of algae), a minimum of seven replicate screens are needed to achieve a reasonable estimate of cell abundances (Tester et al., 2014). Fernández-Zabala et al. (2022) subsequently used the AS method with seven replicates per sampling location to examine changes in the distribution of *Gambierdiscus* and BHAB species with depth. This sampling regime avoided the issues associated with the rapid variation in macrophyte composition and abundance over the 20 m depth profile examined in their study and yielded a coefficient of variation <50% (Cohu and Lemeé, 2012; Fernández-Zabala et al., 2022). For more abundant species such as *Prorocentrum* (>500 cells g^{-1} wet weight of algae) only three replicates per site are necessary to achieve abundance estimates with a CV of <100%. Collectively, the AS studies show a variety of benthic dinoflagellate species can be sampled quantitatively using this method if sufficient numbers of replicates are included in the sampling protocol. In studies where the AS collection method was reported as less satisfactory (Parsons et al., 2017, 2021), inadequate replicate numbers and disregard for the original sampling protocol were the likely causes.

Yong et al. (2018) and Lee et al. (2020) also used the AS method to determine the microhabitats where benthic HABs were most abundant. They found highest densities of *Gambierdiscus* in microhabitats dominated by turf algae, hard coral and, to a lesser extent, fleshy macroalgae. *Ostreopsis* exhibited a preference for the same microhabitats as *Gambierdiscus* in addition to microbial mats. *Prorocentrum* were the numerically dominant species followed by *Ostreopsis, Amphidinium, Coolia*, and *Gambierdiscus*. In the same region, Mohammad-Noor et al. (2016) examined a lagoonal system on Dinawan Island, Malaysia and found the most abundant benthic dinoflagellate species on macrophytes were also the most abundant dinoflagellate species in the water column. In the Mediterranean, Mangialajo et al. (2008) reporedt a correlation between the maximum *Ostreopsis* cell concentrations on macrophytes and in the water column and a similar trend was noted by Vila et al. (2001) based on monthly sampling.

3. Advantages and disadvantages of the macroalgal and artificial substrate collection methods

The macrophyte and screen methods have the following advantages and disadvantages.

3.1. Advantages of the macrophyte sampling method

- It is not necessary to return to the sampling site after 24 h to retrieve samples.
- There is a considerable literature based on this method that has served the community in assessing presence and absence of BHABs.
- In the context of food web research, sampling macrophytes is essential, but so too, are AS samples so results can be compared across studies.
- Some long term BHAB datasets have been established using macrophytes and while it is reasonable for them to continue, they could serve as the bridge to using AS methods in the future. Dual sampling using both macrophytes and AS methods could provide these programs with the confidence they need to discontinue the macrophyte method.

3.2. Disadvantages of the macrophyte sampling method

- Data have shown only cell counts obtained from species with equivalent surface area to wet weight ratios are directly comparable (Lobel et al., 1988). The possibility of cells settling on a macrophyte is greater if it has a higher surface area. Despite this, few investigators go through the tedious process of measuring surface area of different macrophytes found at sampling sites and then normalizing cell densities to surface areas. As Lobel et al. (1988) noted, this invalidates most inter-site comparisons or any time series during which cells were collected from different macrophytes.
- It is difficult to meet the assumption that densities of BHAB cells collected from a preferred macrophyte(s) reflect the overall population at the sampling site. For example, studies have shown the majority of *Gambierdiscus* cells are associated with turf algae in some environments and not the co-occurring macrophytes (Mustapa et al.,

2019; Lee et al., 2020). Sampling only macrophytes would provide a biased estimate of the overall density of BHAB cells.
- Because macrophyte distributions are often variable in time and space, the sampling design in time series experiments may require significant readjustments with temporal shifts in the macroalgal species composition and abundance. The target macrophyte may be absent during part of the year making it impossible to implement statistically sound sampling protocols.
- The macrophyte method precludes sampling if macrophytes are absent, as was the case for *Gambierdiscus* in habitats dominated by turf algae (Lee et al., 2020) or *Ostreopsis* present on manmade structures (Cohu et al., 2014).
- Samples from macrophytes may be mucus covered and littered with silt, sand and debris that cannot be effectively removed by sieving. This causes greater difficulty in counting cells or successfully completing in situ hybridization assays. Inhibitors contained in debris and extracted during the DNA isolation process can also inhibit species-specific qPCR assays for estimating cell abundance.
- Repeated sampling can damage or deplete the population of the host macrophytes.
- Macrophytes cannot be sampled in certain parks or reserves where collecting is prohibited.

3.3. The advantages of the artificial substrate (AS) method include

- AS provides a predictable, consistent method of normalizing cell densities on the basis of surface area.
- In complex habitats, BHABs recruit to AS from the different, naturally occurring substrates over 24 h thus providing integrated cell abundance estimates.
- AS supports statistically robust, repeatable, random sampling designs.
- AS can be used in every habitat regardless of the substrates present.
- AS makes no assumptions about which substrate is most representative of the overall densities of benthic dinoflagellates.
- AS provides cleaner samples that are easier to count and yield more consistent results when analyzed using species-specific molecular assays for identification.
- The AS method is inexpensive and simple to use.
- AS sampling is non-destructive to the habitat and does not denude macrophyte populations making it applicable for use in parks and reserves where removing macrophytes is prohibited.

3.4. Disadvantage of the AS method

- The major disadvantage of the AS method is the need to deploy and subsequently retrieve the screens 24 h later. This increases the time and expense required to obtain samples. The requirement for a 24 h sampling period using the AS method may be due to a diel pattern in the frequency at which *Gambierdiscus* enter the water column and then resettle (Tester et al., 2014; Fernández-Zabala et al., 2019).

4. Settling of benthic dinoflagellates cells on substrates

Another issue relevant to the use of the AS method is a question of preference for certain macroalgae by *Gambierdiscus* and other BHABs. The term "*preference*" has the inherent implication that cells are actively seeking certain macroalgal species. Distinguishing between random settling of BHAB cells on surfaces with the greatest area versus active selection for specific macrophytes (preference) has profound implications for our understanding of BHAB biology and where and how to implement sampling regimes for unbiased samples.

4.1. Field studies

One of the first quantitative field studies to determine if *Gambierdiscus* cells were preferentially settling on one macroalgal species versus another was conducted by Lobel et al. (1988). They used *Dictyota* (Ochrophyta) and *Galaxaura* (Rhodophyta) and found *Gambierdiscus* densities were typically four times higher on *Dictyota* compared to *Galaxaura*. In contrast, when the cell abundances were normalized to surface area (*Dictyota* has ~3–4 times more surface area per unit biomass compared to *Galaxaura*), *Gambierdiscus* densities per unit surface area were equivalent for the two species. Lobel et al. (1988) interpreted their results to mean, "*active preference is unlikely*".

Numerous other field studies have reported observations consistent with *Gambierdiscus* randomly settling on substrates based primarily on the available surface area and not a result of an active preference for specific macrophytes or other substrates (Nishimura et al., 2018). Taylor (1985) noted *Gambierdiscus* are found on "*many species of red, green and brown algae, but its greatest preference is for finely branched or tufted forms*". Bomber et al. (1989) found the abundance of *Gambierdiscus* was positively correlated with macroalgal surface area, with *Heterosiphonia gibbesii* (Rhodophyta), which exhibited the greatest surface area to volume (SA/V) ratio supporting the highest cell densities. When sampling *Gambierdiscus* densities on turf and macroalgae at 21 sites in habitats along the coast of Queensland, Australia, Gillespie et al. (1985) reported "*… almost every species of macroalgae sampled during this study supported a population of G. toxicus [=Gambierdiscus* spp.*], suggesting the organism is opportunistic in regard to macroalgal substrate*".

In a survey of different substrate types in Hawaii, Parson and Preskitt (2007) reported *Gambierdiscus* spp. did not exhibit a preference for any of the twelve host macroalgal species examined, but it was more abundant on species with microfilamentous morphology (70 +/- 119) compared to those with microfilament (11 +/- 42), microblade (24 +/- 57) or macroblade (2 +/- 5) morphologies. Their findings supported microfilamentous turf algae as a significant source of the *Gambierdiscus* entering the piscivorous food web. More recently, Bravo et al. (2020) published that filamentous macrophytes were the preferred substrates for all benthic dinoflagellate genera in the Canary Islands. Kim et al. (2021) examined the relationship between SA/V and cell densities of benthic dinoflagellates from macrophytes collected from Jeju Island, Korea. They reported both the number of genera (*Ostreopsis*, *Gambierdiscus, Amphidinium, Coolia* and *Prorocentrum*) and the number of cells increased as SA/V ratios of the macrophytes increased. They concluded the SA/V ratio of the macroalgal substrates, as well as other environmental factors, including water temperature, salinity and turbulence affected the distribution patterns of epiphytic dinoflagellates. For *Gambierdiscus* and *Ostreopsis*, most macroalgae appeared to be acceptable substrates while the macroalgae with the greatest surface area per unit biomass or volume supported the highest cell densities.

4.2. Literature survey of substrate types supporting the highest density of benthic dinoflagellates

The idea of macrophyte species with the greatest surface area per unit biomass or volume supporting the highest cell densities of benthic dinoflagellates is further supported by a survey of 75 articles reviewed for this study (Table S1). We collected data on which macrophytes had the highest *Gambierdiscus* cell densities. The number of macrophyte species sampled across the various studies ranged from one to >20 so the data in each study were not equally representative of all the substrates in the environment. However, of the macrophytes sampled, higher densities were found on those species with greater surface area per unit biomass. The most commonly reported substrates with the highest *Gambierdiscus* densities, in order, were Rhodophyta, Ochrophyta, Chlorophyta and turf algae (Fig. 3; Table S1). Notably, less frequent reports of turf algae in the literature survey probably represent an underestimate of their importance. These multispecies turfs, with their complex morphologies and high surface area to biomass ratios, represent the substrate most likely to harbor dense *Gambierdiscus* populations (Parson and Preskitt, 2007; Mustapa et al., 2019; Lee et al., 2020; Bravo

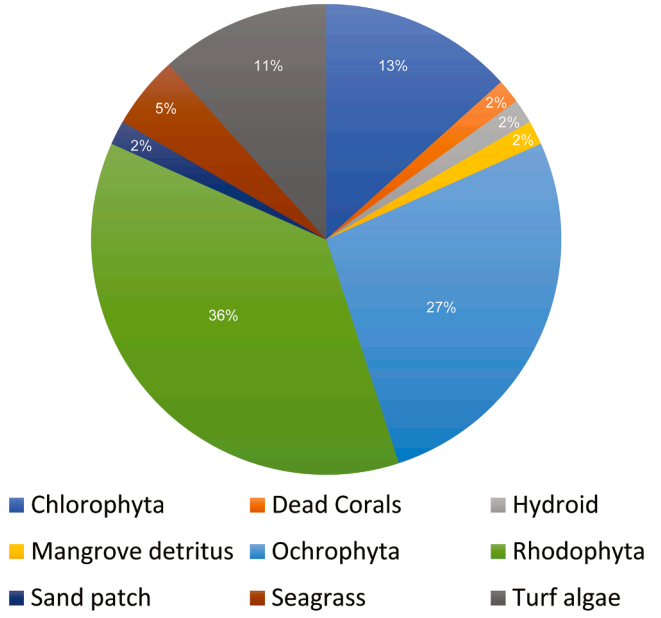

Fig. 3. The macrophytes and other substrates reported as having the highest *Gambierdiscus* cell abundances in each of 75 published studies (See Table S1).

et al., 2020; Holmes et al., 2021). The literature review (Table S1) substantiated the findings of no apparent pattern in active *Gambierdiscus* preference for specific macrophytes.

4.3. Potential effects of biotic interactions

There is a small, but increasing, body of literature on how biotic interactions may contribute to distribution of benthic dinoflagellates on macroalgae. Interactions may be positive if benthic dinoflagellate cells benefit nutritionally from exudates produced by the macrophyte or the exudates limit the growth of competing microalgae or bacteria. If benthic dinoflagellates could select those macrophytes, a positive growth advantage might result. Alternatively, the relationship could be negative, if macrophyte exudates inhibit growth or were toxic. Positive growth of *Gambierdiscus* species in the presence of certain macroalgae was reported by Grzebyk et al. (1984) and Mustapa et al. (2019). In the later study, though the cells grew well in the presence of the various macroalgae, no significant correlation was found between the percent of *Gambierdiscus* cells attached to the macroalgae and the resulting *Gambierdiscus* growth rate. This suggests for the experimental conditions used, attachment to any of the four macroalgal species tested failed to provide a growth advantage.

In addition to these observations, numerous other laboratory studies have shown either neutral or negative effects on growth or survival of benthic dinoflagellates when exposed to various Chlorophyta, Ochrophyta, and Rhodophyta species (Nan et al., 2004; Wang et al., 2007; Tang and Gobler, 2011; Accoroni et al., 2015). These studies must be viewed cautiously, however, because many involved exposing benthic dinoflagellate species to extracts from whole macrophytes which may not accurately represent the suite of exudates released at the surface of the macroalgae (Jeong et al., 2000; Jin and Dong, 2003; Alamsjah et al., 2005; Kim et al., 2006; Tang and Gobler, 2011; Accoroni et al., 2015). Even in studies which used pieces of macroalgae, some substances not in the intact macroalgae may leak into the media. This is supported by the relatively high colonization of *Dictyota dichotoma* (Ochrophyta) by *Ostreopsis* cf. *ovata* in the field despite extracts of *D. dichotoma* negatively impacting *O.* cf. *ovata* growth (Ternon et al., 2020).

These sometimes-conflicting results are likely due to the difficulty in accurately replicating field conditions (water movement, grazing losses, nutrient fluxes, variable light, water-air gas exchange) in vitro studies. Sustaining macroalgae in a healthy condition long enough to allow immigration and emigration of benthic dinoflagellate cells to reach equilibrium is challenging. Given these limitations, it is difficult to interpret laboratory studies, especially if the results were not normalized to surface area. As a result, there is no compelling experimental evidence *Gambierdiscus*, and perhaps other benthic dinoflagellate species, are host dependent (Mustapha et al., 2019). Overall, the observations documented in this review confirm SA/V is a primary determinant of settling frequency, with highest benthic dinoflagellate densities occurring on the most structurally complex (highest SA/V ratio) macroalgae. This argues for the unbiased nature of using artificial substrates as a collection method to provide precise, comparable surface area-based cell estimates in complex habitats.

5. Cell-based monitoring strategies for assessing ciguatera risk

A primary goal of HAB research is to inform the development of monitoring strategies to reduce adverse health and economic impacts. Ideally, in the case of CTXs produced by some *Gambierdiscus* species, which cause ciguatera fish and shellfish poisoning (CP), the seafood entering the market would be tested directly. However, the lack of toxin standards, the expense of sample preparation and cost of analytical equipment preclude routine, large-scale testing. This has stimulated interest in the development of cell-based early warning systems (Rott, 1981; Vassalli et al., 2018).

Groundbreaking research in French Polynesia (FP) provided data on the effects of temperature on *Gambierdiscus* abundance and CP risk (Chinain et al., 1999; Chateau-Degat et al., 2005). The peak number of CP illnesses were reported three months after a series of blooms often exceeding 1000 cells g^{-1} wet weight. Subsequent efforts showed highly toxic *Gambierdiscus* species comprising as little as 0.16–6.3% of the total bloom population, may produce as much toxin as the other species combined (Litaker et al., 2017; Pisapia et al., 2017; Rossignoli et al., 2020).

The importance of both cell abundance and species composition was well illustrated by Darius et al. (2017). On a small French Polynesian island, the gastropod *Tectus niloticus* became ciguatoxic and caused CP only in locations where *Gambierdiscus* populations were dominated by *G. polynesiensis*, the most toxic *Gambierdiscus* species in the Pacific Ocean. (Darius et al., 2017). The snails became toxic when cell abundances were elevated (>2900 cells g^{-1} wet weight, 82% of which were *G. polynesiensis*). At two other bays on the island where *Gambierdiscus* cell concentrations were lower (<420 cells g^{-1} wet weight) and dominated by low-toxicity species, the snails were not toxic.

Recently, species-specific quantitative polymerase chain reaction (qPCR) assays have been developed that can identify many *Gambierdiscus* species (Vandersea et al., 2012; Nishimura et al., 2016; Smith et al., 2017; Litaker et al., 2019; Kretzschmar et al., 2019; Gaiani et al., 2021). Though still requiring specialized equipment, qPCR assays are being performed routinely in local and regional public health and contract laboratories and will become increasingly accessible with time. Additionally, new recombinase polymerase amplification methods for species detection are being developed (Gaiani et al., 2021). This information served as the basis for the recently proposed BHAB early warning guidance for UNESCO programs, which includes using AS to collect cells and qPCR to identify and quantify the species present (Technical Guidance for the Implementation of Early Warning Systems for Harmful Algal Blooms, FAO, WHO, IAEA, 2022).

6. Sampling in high turbulence environments

The efficacy of AS in high turbulence regions has yet to be fully tested. In one study, Argyle (2018) detected low densities of *Gambierdiscus* from macrophyte samples found within crevasses and pools in a rock platform along the coast of Tonga subjected to high wave action. Here, AS deployed in the same locations failed to collect *Gambierdiscus*

cells. In contrast, *Gambierdiscus* concentrations were high on both the natural substrates and AS deployed in sheltered, shallow, subtidal sites. Some laboratory data indicated *Gambierdiscus* cells remain attached more firmly under turbulent conditions (Nakahara et al., al.,1996), but this has not been field tested. Resolving the issue of how well screens perform in high turbulence areas will require 10 or more replicates to obtain statistically relevant data given the low cell densities found in these environments. Such studies should include quantitative estimates of turbulence or flow rates as was done by Fernández-Zabala et al. (2022) and who reported lower *Gambierdiscus* cell abundances in shallow areas (<5 m) subjected to higher flow rates compared to samples collected at greater depths.

7. Implementation: recommendations for artificial substrate method

- For monitoring purposes *Gambierdiscus* cell densities normalized to surface area are a requisite.
- Normalization of cell counts to surface areas provides an optimal basis for comparisons among sites and over time.
- Surface areas of the screens should be carefully calculated (based on the number and circumference of the fibers comprising the screen as described in Tester et al., 2014) and not just the outer dimensions of the screen. The mesh size of the screen is important and should be standardized in the range of 1.0–1.5 mm (Jauzein et al., 2016).
- Adequate sample replication is required to obtain statistically significant cell estimates. The number of samples needed increases when cell abundance is low.
- Validating a monitoring program for a specific location requires an initial survey to compare methods. Cell counts from macroalgal samples or other substrates should be compared with those from adjacent screen samples incubated for 24 h. These comparisons, however, will only be statistically relevant in instances where BHAB cell concentrations vary over a range from ~400 to >10,000 cells 100 cm^{-2} (see Fig. 2). Concentrations of fewer than <400 cells 100 cm^{-2} (<100 cells g^{-1} wet weight algae) represent background cell levels that are highly variable. At these cell concentrations, a high number of replicates will be required to have a coefficient of variation $\leq$ 100%.
- Due to their many advantages, collection of benthic *Gambierdiscus, Ostreopsis, Prorocentrum, Coolia*, and *Amphidinium* species should be carried out using artificial screens supported by a frame (Tester et al., 2014; Jauzein et al., 2018; Fernández-Zabala et al., 2019).

8. Summary

The increased numbers of adverse incidents associated with BHABs such as *Gambierdiscus* and *Ostreopsis* have generated strong research interests in the last decade, particularly with regard to taxonomy, toxin characterization and habitat requirements. With recommendations going forward at the request of FAO, WHO, IOC and IAEA for monitoring protocols that can serve as early warning systems to identify increased abundances of toxic benthic species, implementation of a standardized sampling protocol is essential. Given this challenge, we present numerous advantages of using artificial substrates versus sampling macrophytes for estimating *Gambierdiscus* species densities. This approach is bolstered by evidence demonstrating that higher *Gambierdiscus* cell densities are found on substrates with the greatest surface areas and not because of active selection or preference of one macrophyte species over another for *Gambierdiscus* settlement. Cell abundances normalized to surface area rather than biomass of host macrophytes allows comparisons among sampling locations, over seasons and among studies, characterizing temporal bloom dynamics. Because low densities of highly toxic species can contribute disproportionately to the flux of ciguatoxins into the marine food chain, using artificial substrate collection methods in conjunction with species-specific molecular assays are required to integrate cell-based risk assessments into routine monitoring programs.

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.

Data Availability

Data are included in Supplementary Table S1.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2022.102351.

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