CHAPTER VII

Benthic Dinoflagellate Sampling*

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Introduction

Dinoflagellates are one of the most abundant and diverse groups of the Alveolata. There are more than 2,000 recognized species, which inhabit all freshwater and marine environments (Guiry and Guiry 2016). Often, they contribute significantly to the overall primary productivity of a system or of the microbial food web (Hacket et al. 2004). Approximately five percent of dinoflagellate species are toxic or otherwise harmful, reaching sufficient densities to cause animal mortalities, disruption of normal ecosystem functions and cause human illnesses. Classically, the most studied of these "harmful algal bloom" (HAB) dinoflagellates are planktonic species like those belonging to the genera *Alexandrium* or *Karenia*. In recent years there has been increased recognition that some benthic dinoflagellate species also produce toxins or other bioactive compounds which adversely affect human and animal health (Yasumoto et al. 1987). In this chapter, these harmful benthic species will be termed *BHAB dinoflagellates* (GEOHAB 2012).

In comparison to the planktonic species, the distribution and ecology of BHAB dinoflagellates is poorly understood. Much of this uncertainty is attributable to the marginal environment in which benthic dinoflagellates live—neither suspended in the water column nor buried in the sediment. The benthic species are instead associated with three dimensional substrates or with the sediment-water interface. The terminology used to describe BHAB dinoflagellates reflects this uncertainty. For instance, the term *periphyton* is used by freshwater researchers to describe the community of bacteria, micro- and macroalgae, protozoa and other associated organisms growing firmly attached to submerged macrophytes. Marine researchers have most commonly used the term *epiphyton* to describe similar biota on seagrasses and macroalgae. BHAB dinoflagellates, like many other flagellated or ciliated protists, are frequently motile and may not be truly attached to substrates in the same manner as freshwater periphyton (Nakahara et al. 1996). Therefore, other terminology may be more appropriate to describe the association

In Steidinger, K.A. and Meave del Castillo, M.E. [Eds.] *Guide to the Identification of Harmful Microalgae in the Gulf of Mexico.* © 2018

between dinoflagellates and various substrates. For instance, some researchers studying surface associated biota have distinguished *true epiphytes* from *pseudoepiphytes* based on the degree of attachment. True epiphytes are defined as those with an obvious physical attachment to macrophytes, while pseudoepiphytes are merely associated with the surface (Goldsborough et al. 1986, Comte et al. 2005). Thus far, these two groups have been defined largely by the sampling methods used to collect them. Pseudoepiphytes are removed from substrates by gentle agitation or shaking whereas true epiphytes require scraping or brushing to remove them from substrates (Comte et al. 2005). An equivalent term for pseudoepiphyton is *metaphyton* (Behre 1956). Based on the observation that BHAB dinoflagellates can be displaced readily by shaking macroalgae (see below), they are operationally defined as members of the metaphyta. Other terms used to describe BHAB dinoflagellates include epibenthic, epipsammic (attached to sand grains; also episammonic, Skinner et al. 2009) and tychoplanktonic (benthic biota temporarily suspended in the water column, Steidinger and Baden 1984). In this chapter, the term *benthic* will be used as a general term to encompass all of these definitions.

Part I. The organisms

Gambierdiscus

The dinoflagellate genus *Gambierdiscus* Adachi et Fukuyo (Goniodomataceae) is best known for its association with ciguatera fish poisoning (CFP), a food-borne illness affecting humans, marine resources and local economies worldwide (Berdalet et al. 2016). Some *Gambierdiscus* species produce ciguatoxins (CTXs), lipophilic compounds that are readily transferred through the food web from algae, to herbivorous fish, to carnivorous fish and ultimately to humans (Table 1). Multiple structural forms of the toxins (congeners) with varying toxicities have been identified (Murata et al. 1989, Scheuer 1996). The possibility also exists that co-occurring benthic dinoflagellates (*Fukuyoa*, *Ostreopsis*, *Prorocentrum*, *Coolia*, and *Amphidinium* species) may play a role in CFP, but toxins or derivatives from these dinoflagellates have not, as yet, been recovered from fish tissues. Ciguatoxins negatively affect several types of mammalian cells, most notably impacting nerve transmission via activation of voltage dependent sodium channels (Lombet et al. 1987, Lewis et al. 1991). Humans develop CFP after consuming tropical or subtropical fish containing sufficiently high concentrations of CTX. Typical symptoms include diarrhea, vomiting (gastrointestinal), numbness or tingling of the mouth and digits, ataxia, muscle aches (neurological) and irregular heartbeat, reduced blood pressure and paralysis (cardiovascular) (Friedman et al. 2008). Mitigation of CFP by health organizations has been complicated by difficulties with diagnosing the illness, inconsistent or nonexistent reporting and the wide degree of spatial and temporal variability among CFP incidences (Tester et al. 2010). Though some of the symptoms of CFP were recognized as early as the 1500s (Halstead 1967), the genus *Gambierdiscus* was not formally described until 1979 (Adachi and Fukuyo 1979). Currently, the genus *Gambierdiscus* has at least fifteen described species and several ribotypes (Litaker et al. 2009, Fraga et al. 2011,

2016, Fraga and Rodriguez 2014, Nishimura et al. 2014, Smith et al. 2016, Kretzschmar et al. 2017, Rhodes et al. 2017). There are also three sister species in the new genus *Fukuyoa* (Gómez et al. 2015).

Ostreopsis

Dinoflagellates in the genus *Ostreopsis* Schmidt (Ostreopsidaceae) include nine extant species occurring in tropical, subtropical and temperate environments (*O. belizeanus*, *O. caribbeanus*, *O. heptagona*, *O. labens*, *O. lenticularis*, *O. marinus*, *O. mascarenensis*, *O. ovata*, and *O. siamensis*) (Accorini & Totti 2016). Because of unresolved issues concerning morphological differences and lack of genetic data for the holotype specimens of *O. siamensis* and *O. ovata*, reference of these two species as *O.* cf. *ovata* and *O.* cf. *siamensis* has been adopted pending more data (Penna et al. 2005, 2010).

Ostreopsis species produce a series of water soluble, highly toxic compounds including palytoxins (PLTXs), mascarenotoxins (McTXs) and ovatoxins (OvTXs) (Amzil et al. 2012, Accorini & Totti 2015) (Table 1). In humans and other mammals, PLTX and associated toxins are powerful vasoconstrictors targeting the ATPase Na+/K+ pump, a transmembrane enzyme that plays a role in maintaining the resting potential of nerve, muscle and heart cells (Usami et al. 1995, Rhodes et al. 2002, Tichadou et al. 2010, Rossini and Bigiani 2011). The result of these potent neurotoxins is palytoxicosis, characterized by many symptoms including salivation, abdominal cramps, nausea, severe diarrhea, muscle spasms and breathing difficulties, followed by death in the most severe cases (Table 1) (Yasumoto et al. 1986, Alcala et al. 1988, Yasumoto 1998). Of particular concern in coastal areas of the Mediterranean are toxin-containing aerosols responsible for febrile respiratory syndromes as well as respiratory and skin irritations (Gallitelli et al. 2005, Penna et al. 2005, Brescianini et al. 2006, Ciminiello et al. 2006, Illoul et al. 2012).

In the last decade, *Ostreopsis* HABs have seriously impacted coastal flora and fauna and have caused widespread human illness. In the warm subtropical waters of the Mediterranean Sea, blooms of *O.* cf. *ovata* have plagued coastal areas, causing mass mortalities as well as various sublethal impacts to benthic and planktonic invertebrates and microalgae, fish and indirectly, terrestrial vertebrates (Brescianini et al. 2006, Ciminiello et al. 2006, Vale and Ares 2007, Shears and Ross 2009, Totti et al. 2010, Faimali et al. 2011, Accorini et al. 2015). Palytoxins and associated compounds have also been found in the water column, in floating clumps of detritus and cells and in aerosols dispersed by the wind (Amzil et al. 2012). There is growing evidence that toxins produced by *Ostreopsis* species can be transmitted through the food web to humans via consumption of fish, shellfish, crabs and urchins (Rhodes et al. 2000, Aligizaki et al. 2008, 2011, Deeds and Schwartz 2010, Amzil et al. 2012).

Table 1. Benthic harmful algal bloom (BHAB) dinoflagellates and associated toxins.

BHAB Genera	**Toxins**	**Notes**	**Reference**
Gambierdiscus *Fukuyoa*	Ciguatoxins Gambiertoxins Maitotoxins Gambierol	Gastrointestinal, neurological and cardiovascular symptoms including numbness and tingling of hands and feet, temperature reversal sensations, difficulty balancing, low heart rate and blood pressure, rashes In extreme cases ciguatera fish poisoning can cause death due to respiratory failure	Yasumoto et al. 1977b Yasumoto et al. 1979b Bagnis et al. 1980 Gillespie et al. 1985 Babinchak et al. 1986 McMillan et al. 1986 Durand-Clement 1987 Bomber et al. 1988b Bomber and Tindall 1988 Bagnis et al. 1990 Holmes et al. 1991 Lewis and Holmes 1993 Holmes et al. 1994 Chinain et al. 1999 Estacion 2000 Guzmán-Pérez and Park 2000 Ghiaroni et al. 2005 Hamilton et al. 2010 Rhodes et al. 2010 Roeder et al. 2010 Schlumberger et al. 2010 Gómez et al. 2015
Ostreopsis	Palytoxin Palytoxin-like toxins Ostreocines Mascarenotoxins Ovatoxins	Dermal, ocular irritation and respiratory illness due to inhalation of aerosols Rhabdomyolysis after eating fish contaminated with palytoxins	Riobó et al. 2004 Ciminiello et al. 2006 Cagide et al. 2009 Ciminiello et al. 2010 Deeds and Schwartz 2010 Amzil et al. 2012 Crinelli et al. 2012 Pezzolesi et al. 2012
Prorocentrum	Okadaic Acid Dinophysistoxin Yessotoxins Domoic Acid	Diarrheic shellfish poisoning Ribotoxic stress	Grzebyk et al. 1997 Barbier et al. 1999 Pan et al. 1999 Draisci et al. 2000 Bouaïcha et al. 2001 Bravo et al. 2001 Kurisu et al. 2003 Foden et al. 2005 Paz et al. 2008 An et al. 2010 Hu et al. 2010 Murray et al. 2010 Varkitzi et al. 2010 Li et al. 2012 Korsnes. et al. 2014 López-Rosales et al. 2014

BHAB Genera	Toxins	Notes	Reference
Coolia	Cooliatoxin Cooliatin (dioxocyclononane)	Symptoms similar to those produced by yessotoxins Hypothermia and respiratory failure in mice	Nakajima et al. 1981 Holmes et al. 1995 Rhodes and Thomas 1997 Liang et al. 2009
Amphidinium	Cytotoxic Macrolides Polyketides Polyhydroxyls Extracellular Polymers (EPS)	Antifungal and hemolytic activity Implicated as a causative agent in human ciguatera	Kubota et al. 2001 Kobayashi et al. 2003 Kobayashi and Tsuda 2004 Tsuda et al. 2005 Baig et al. 2006 Zimmermann 2006 Huang et al. 2009 Mandal et al. 2011 Pagliara and Caroppo 2012

Prorocentrum

The genus *Prorocentrum* (Prorocentraceae) was erected by Ehrenberg with *P. micans* as the type species. Currently, there are about 80 accepted species in the genus with at least twelve more pending descriptions (Hoppenrath et al. 2011, Guiry and Guiry 2016). Many *Prorocentrum* species inhabit marine or brackish environments and are frequently abundant in tropical, subtropical and temperate waters (Nagahama et al. 2011). Three freshwater species from Europe (Delmail et al. 2011) and Australia (Croome and Tyler 1987) also have been described. Some species, such as *Prorocentrum micans, P. gracile* and *P. texanum* are predominantly planktonic, while others are mainly benthic (e.g., *P. leve*, *P. lima, P. steidingerae*) (Faust et al. 2008, Hoppenrath et al. 2013, David et al. 2014, Hoppenrath et al. 2014, Gómez et al. 2017). Like the rest of the BHAB genera, the genus *Prorocentrum* is currently in flux with many new species described in recent years as more detailed molecular information has become available.

The evidence for toxicity in *Prorocentrum* species is also being revisited. The most widely recognized toxic benthic species in the genus is *P. lima*, which produces okadaic acid (OA) as well as domoic acid (DA) (Table 1); it has been implicated as a cause of diarrheic shellfish poisoning (DSP) in humans (Heredia-Tapia et al. 2002). The acute symptoms of DSP include diarrhea, nausea, vomiting and abdominal pain. Outbreaks have been documented worldwide and are associated with the consumption of mussels, scallops, or clams tainted with OA, its analogs or derivatives (Gestal-Otero 2000). However, the general contribution of *Prorocentrum* species in DSP outbreaks remains unclear. In general, the presence of DSP toxins in shellfish is most closely linked with planktonic *Dinophysis* blooms. Benthic *Prorocentrum* species, in contrast, rarely have been directly implicated in such events (Marr et al. 1992, Quilliam et al. 1993, Vale et al. 2009).

Okadaic acid produced by *Prorocentrum* species is known to be a potent tumor promoter (Dounay and Forsyth 2002). Various *Prorocentrum* species also produce >20 bioactive esters and polyketides (Hu et al. 2010, Li et al. 2012, Vilches et al. 2012). In contrast, planktonic *Dinophysis* species produce okadaic acid as well as dinophysistoxins (DTX-1 and DTX-2), which inhibit protein phosphatases PP1 and PP2A significantly affecting transcription and other cellular functions (Blanco et al. 2005, Marcaillou et al. 2005, Dominguez et al. 2010, Gerssen et al. 2010, Fernández et al. 2014). To date, at least 35 naturally occurring derivatives of OA and DTXs have been identified (Table 1).

The production of okadaic acid and other toxins is now viewed as a taxonomic character in *Prorocentrum* species. Toxicity has been demonstrated in the closely related species in *Prorocentrum* clade 2, including *P. arenarium* (= *P. lima*), *P. belizeanum*, *P. concavum*, *P. faustiae*, *P. hoffmannianum*, *P. leve*, *P. lima*, and *P. maculosum* (Faust 2002, Faust et al. 2008, Murray et al. 2009, An et al. 2010, Hu et al. 2010). Based on their phylogenetic position among these toxic species, *P. bimaculatum* and *P. consutum* could be considered as potential toxin producers as well (Chomérat et al. 2012).

Coolia

Coolia Meunier 1919 is a genus of predominantly benthic/planktonic thecate dinoflagellates that co-occur with *Gambierdiscus* and other toxic species. Originally described from oyster beds in Belgium (Meunier 1919), *Coolia* cells were later found to be associated with macroalgae in the tropical Atlantic and Pacific (Fukuyo 1981, Carlson and Tindall 1985). The wide geographic range of *C. monotis*, which seemingly occurred in both tropical and temperate locations, was shown to include a number of cryptic species (Leaw et al. 2016). There are seven extant species: *Coolia monotis*, *C. tropicalis*, *C. canariensis*, *C. areolata*, *C. malayensis*, *C. santacroce* and *C. palmyrensis*, although there has been some contention about strain/species boundaries (Leaw et al. 2010, Karafas et al. 2015, Karafas & Tomas 2015, Guiry and Guiry 2016, Leaw et al. 2016).

There has been contention about the toxicity of this genus as well. Holmes et al. (1994) found a dense population of *C. monotis* in a bay in Queensland, Australia that appeared to be responsible for the majority of ciguatera-like intoxications in that area. A toxic compound isolated from cultured cells, cooliatoxin, was found to be highly toxic to mice (Holmes et al. 1994) (Table 1). Cooliatoxin was described as structurally similar to yessotoxin, a compound causing shellfish toxicity that is produced by dinoflagellates in the genera *Protoceratium*, *Lingulodinium* and *Gonyaulax* (Howard et al. 2009, Gerssen et al. 2010). *Coolia* species have therefore been considered a potential cause of seafood poisoning (Faust 1995). Other evidence for toxicity in *Coolia* includes hemolytic activity in Japanese isolates of *C. monotis* (Nakajima et al. 1981) and *Artemia* bioassay toxicity in Portuguese *Coolia* strains (Fonseca Hinzmann 2005). Similarly, Liang et al. (2009) found a new dioxocyclononane compound termed cooliatin that was present in *C. monotis* from the South China Sea (Table 1).

In contrast, several authors have reported that *Coolia* strains/species appear to be nontoxic. For example, Fraga et al. (2008) found no evidence of toxins in strains of either *C. monotis* or *C. canariensis*. Similarly, no yessotoxin analogs were detected in European or Mediterranean strains of *C. monotis* (Riobó et al. 2004, Penna et al. 2005, Laza-Martínez et al. 2011). Other authors have reported *Coolia* strains/species from both the Atlantic and Pacific showed no evidence of toxicity in mouse bioassays (Yasumoto et al. 1980, Nakajima et al. 1981, Tindall et al. 1984, Lee et al. 1989). Subsequently, five more distinct analogs of yessotoxin were characterized chemically from *C. malayensis* (Wakeman et al. 2015). Taken together, the toxicity data indicate interspecific differences in toxicity/bioactivity as well as regional differences in strains, although it seems likely that further study may reveal even more species (Rhodes and Thomas 1997, Rhodes et al. 2000, Penna et al. 2005, Jeong et al. 2012, Mohammad-Noor et al. 2004, 2013, Momigliano et al. 2013, Shah et al. 2014).

Amphidinium

Amphidinium Claparède & Lachmann (Gymnodiniaceae) was, until recently, a large and very diverse genus of benthic and planktonic unarmored dinoflagellates. Included in the Order Gymnodiniales, the genus formerly had 50-100 species that were planktonic, surface associated, interstitial or endosymbiotic. These include phototrophic, mixotrophic and heterotrophic species distributed in marine and freshwater environments. Reflecting multiple corrections and re-descriptions that have occurred, the genus is now referred to as *Amphidinium* Claparède et Lachmann, 1859 emend. Flø Jørgensen, Murray et Daugbjerg, 2004, and has a reduced number of species (*sensu stictu*) (Flø Jørgensen et al. 2004). There is still much uncertainty in morphological and phylogenetic species designations for this group.

Amphidinium species produce a number of bioactive compounds that may have a role in bloom formation and toxicity. For example, amphidinolides and colopsinols are two groups of unique macrolides that are produced by *Amphidinium* spp. (Kobayashi et al. 2003, Murray et al. 2012) (Table 1). Amphidinolides have direct biomedical application as a treatment for human tumors (Kobayashi et al. 2003). Other compounds produced by this genus reportedly have a direct role in fish kills. For instance, some strains of *A. operculatum* var. *gibbosum* and *A. carterae* have been observed to produce ichthyotoxins, which may include amphidinols, amphidinolides and caribenolides (Kobayashi et al. 1991, Satake et al. 1991, Bauer et al. 1994, 1995, Paul et al. 1997, Houdai et al. 2001) (Table 1). Some of these compounds also have direct cytotoxic and/or hemolytic activity against fungi, bacteria and other algal cells (Nagai et al. 1990, Satake et al. 1991, Nayak et al. 1997, Ji et al. 2012). The role of *Amphidinium* as a HAB dinoflagellate has been widely recognized because of the production of these compounds (Sampayo 1985, Yasumoto et al. 1987, Tindall and Morton 1998, Echigoya et al. 2005, Huang et al. 2009, Rhodes et al. 2010). In recent years, *A. carterae* has been shown to produce large amounts of extracellular polymer substances (EPS), hypothesized to have a role in bloom formation and possibly fish kills (Mandal et al. 2011). Despite evidence of their harmful characteristics, there have been no reports of direct human health impacts by *Amphidinium* toxins, although the genus has been implicated in ciguatera-like fish poisonings

because of its co-occurrence with *Gambierdiscus* and other potentially toxic benthic species (Bomber and Aikman 1989, Tindall and Morton 1998, Baig et al. 2006).

Part II. Sampling methods

Substrate collection

Because of their common association with three dimensional substrates, BHAB dinoflagellates have most commonly been sampled by collection of the substrates themselves. Typically, dinoflagellate abundances are normalized to substrate mass (cells g^{-1} wet or dry weight), surface area (cells cm^{-2}, cells m^{-2}) or volume (mL^{-1}, cm^{-3}, m^{-3} for sediment) (Taylor and Gustavson 1985, Bomber and Aikman 1989, Tindall and Morton 1998). The most common substrates used to assess BHAB distribution and abundance are macrophytes (macroalgae and seagrasses), lithogenous materials such as dead coral, shells and rocks, sediment and other benthic materials such as plant matter or invertebrates (Kohler and Kohler 1992, Aligizaki and Nikolaidis 2006, Aligizaki et al. 2009, Amorim et al. 2010). Floating or drifting substrates have also been utilized, including macroalgae, flocs of cells and benthic matter and clumps of detritus (Bomber et al. 1988a, Faust 2004, Faust and Tester 2004). In some instances, artificial materials such as rope have been used (Faust 2009).

The biggest advantage to substrate collection is that it is a simple method, often requiring equipment no more sophisticated than a collecting container. Sample collection is typically accomplished by wading, swimming, snorkeling or SCUBA. The main disadvantages of the method involve the availability of sufficient substrate to allow multiple samples and the inherent linkage of cell abundances to factors governing the substrates themselves (see below). More practical considerations include separating the dinoflagellates from substrate material, concentrating cells to densities that are useful and the presence of sediment and other contaminating matter in the resulting cell samples. Although cells can be sampled quantitatively, inter-comparison among different substrates has posed difficulties that have not yet been adequately resolved (see Macrophyte collection below). More detailed information about the most common substrates is presented below.

Macrophyte collection

By far, the most common method for sampling BHAB dinoflagellates is collection of macrophytes, which include both macroalgae and seagrasses. Macrophytes have proven to be convenient source for BHAB dinoflagellates in many tropical and subtropical locations; some of the earliest research on CFP was conducted by collection of macroalgae (see Adachi and Fukuyo 1979). Specimens of macroalgae or seagrass are typically placed in plastic bags or sample jars together with ambient seawater. The cells are separated by shaking the substrate and the sample is sieved to remove large sediment and detritus. The sieved cell sample is then used to establish cultures, for molecular characterization, toxin analysis, and/or to determine cell abundance. Dinoflagellates in a fixed subsample are counted via microscopy and abundance is most commonly reported as cells g^{-1} wet weight (fresh weight) of macrophyte (reviewed by Litaker et al. 2010, Reguera et al. 2011).

For macroalgae, whole thalli or portions thereof are most often collected by hand and specimens are placed into sealable plastic bags or sample jars. Samples should be collected with sufficient ambient seawater to prevent dinoflagellate mortality and physical damage to the algae during transport as well as buffering them from desiccation. For relatively small macroalgae, the entire plant is generally collected, although the holdfast should be removed to limit the amount of sediment in the sample (Fig. 1). For larger algae, a portion may be pinched or cut off the plant. In seagrass samples, an appropriate number of grass blades are usually cut off at the sediment surface avoiding roots or rhizomes.

Regardless of the type of macrophytes that are collected, care should be taken to prevent mortality of the BHAB cells of interest. First and foremost, the samples should be maintained near the ambient water temperature of the collection site and exposure to direct sunlight should be avoided. When multiple collections are made in the field, the best results are generally obtained when samples are placed into a cooler or circulating water bath to maintain the appropriate temperature until they can be processed. Another danger that can cause mortality of BHAB cells is oxygen depletion. This is of particular concern in the tropics, where water temperatures are nearly always high (>25 °C) or during the summer months in more temperate latitudes. To avoid hypoxia sample containers should not be overloaded with macrophyte tissue. As a rule, macrophyte specimens should not take up more than half the volume of the sample container, the remainder of the volume being ambient seawater. Furthermore, BHAB cells should be separated from their substrates as soon as possible after collection. Even a few hours in sealed containers can rapidly deplete oxygen concentrations. If cell abundance is not of direct interest, it may be beneficial to pour off a small amount of the seawater and leave the containers at least partially open to the atmosphere to allow gas exchange. Another reason samples should be processed rapidly is that the assemblage of metaflora and fauna may change with time. Because of the stresses experienced by dinoflagellate cells during collection and transport, the assemblage present when samples are collected may change markedly within hours of collection. For instance, it is not uncommon to observe a diverse assemblage of dinoflagellates immediately after sampling, but then only a few stalwart species or resting

cysts remain after a single day has elapsed. If samples are not processed immediately, they should be kept in the shade in low light with the container lid loosened.

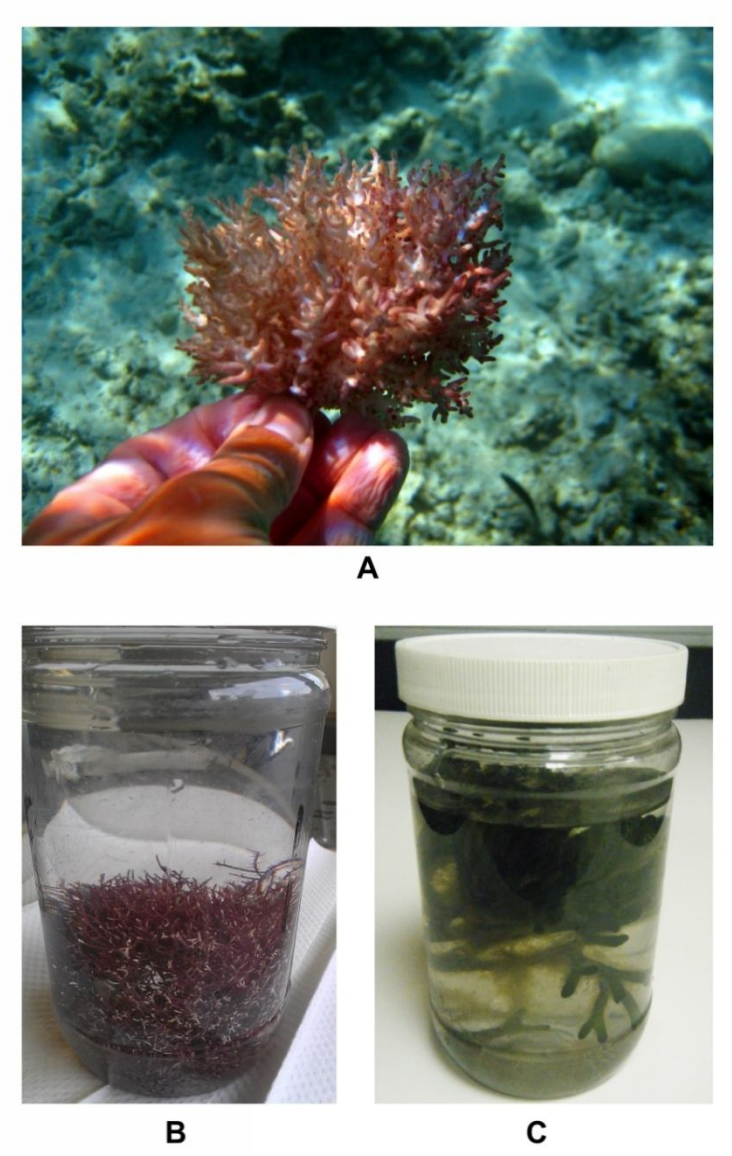


Figure 1. The macrophyte collection method. **A.** Collection of a *Trichogloea* colony by a diver in Hawai'i, http://noaacred.blogspot.com/2010/09/i-wanted-to-be-fireman-or-dentist-or.html. **B.** Sample jar with *Amphiroa* specimen. Credit: NOAA. **C.** Sample jar with *Codium* specimen. Credit: NOAA.

Despite its simplicity and widespread use, the macrophyte method has some substantial disadvantages, especially for quantitative studies dealing with BHAB cell abundance and/or distribution. Among the most problematic is that the desired macrophytes may not occur in the study site(s) of interest or may be present in quantities insufficient to allow replicate sampling. This is particularly troublesome for inter-comparisons among sites or for temporal sampling at the same site (Tester et al. 2014). The dependence upon macrophytes for sampling BHAB dinoflagellates also links the factors governing dinoflagellate distribution with those regulating the distribution of the macrophytes themselves. This covariation may arise from a variety of dinoflagellate-independent factors such as grazing of macrophytes by herbivores, seasonal changes in macrophyte abundance and sediment type or wave energy. Another disadvantage of the macrophyte method is identification of the macroalgae; this may be challenging. Correct identifications are essential if dinoflagellate-substrate interactions are of interest.

Another of the most widely recognized challenges with the macrophyte sampling method involves normalization of cell abundances to some unit allowing comparisons among substrates. Macroalgae possess an array of complex three-dimensional morphologies (Boller and Carrington 2006, Yniguez et al. 2010). As cell abundance is usually normalized to macrophyte mass (cells g^{-1}), comparisons of cell abundance among different morphotypes, genera or macrophyte species are problematic because surface area:mass ratios vary widely (Fig. 2). Researchers have adopted sampling strategies to cope with this problem including limiting sampling to a single macrophyte species (Ballantine et al. 1988), restricting sample collection to a specific season when macroalgae are available (Carlson 1984), restricting sampling to only macrophyte species present at all sampling sites (Richlen and Lobel 2011), focusing on a single site and time (Lobel et al. 1988), or using relative abundance measures such as % total taxa (Bates 2007) or % cover on the host epiphyte (Kersen et al. 2011). Some authors have normalized to arbitrary units of measurement, such as cells per linear unit of stem (Krecker 1939, Levin and Mathieson 1991). These strategies each narrow the particular hypotheses that may be tested as well as their environmental relevance.

The need for standardizing cell abundance to algal surface area (cells cm^{-2}) rather than mass has long been recognized as the most logical solution to these problems (Bomber et al. 1985, Lobel et al. 1988), but the methods for doing so are often complicated and are not uniform. Historically, the most frequent method for quantifying surface area of three dimensional substrates including macroalgae, seagrasses, aquatic plants, terrestrial plant roots, corals and other materials has been direct measurement of substrate dimensions (Bomber et al. 1985). This method involves tedious measurement of stems, leaves, branches and other surfaces, often using geometric shapes that approximate the dimensions of the substrates. However, the direct measurement method is generally possible only for simple substrate morphotypes, such as seagrasses or macroalgae with flattened blade-like thalli, such as *Dictyota* spp. (Lobel et al. 1988). More complex methods have evolved with technological advances from photograph- and photocopy-based image capture techniques (Kokko et al. 1993, Gerber et al. 1994), to computer intensive digital image analysis (Brown and Manny 1985, Lobel et al. 1988, Bradshaw et al. 2007) and laser scanning 3-d tomography (Naumann et al. 2009, Igathinathane

et al. 2010). Quantitative adhesion of microbeads, dyes, detergents or other chemicals have also been utilized to quantify surface area of geometrically complex substrates such as filamentous branching macroalgae (Harrod and Hall 1962, Koppel et al. 1988, Bomber and Aikman 1989, Armstrong et al. 2003). The tradeoff in practicality between the more complex computer intensive methods and simpler, time intensive techniques depends upon the needs of the individual study and whether it occurs in the field or in a modern laboratory. Because of these practical limitations, and despite the disadvantages detailed above, the macrophyte collection method may be the best choice for researchers seeking to assess the presence/absence of BHAB species, or for those primarily interested in characterizing BHAB dinoflagellate diversity. Other methods, such as the artificial substrate method (see below) offer many advantages for inter-site comparisons, spatial-temporal investigations and more robust hypothesis testing.

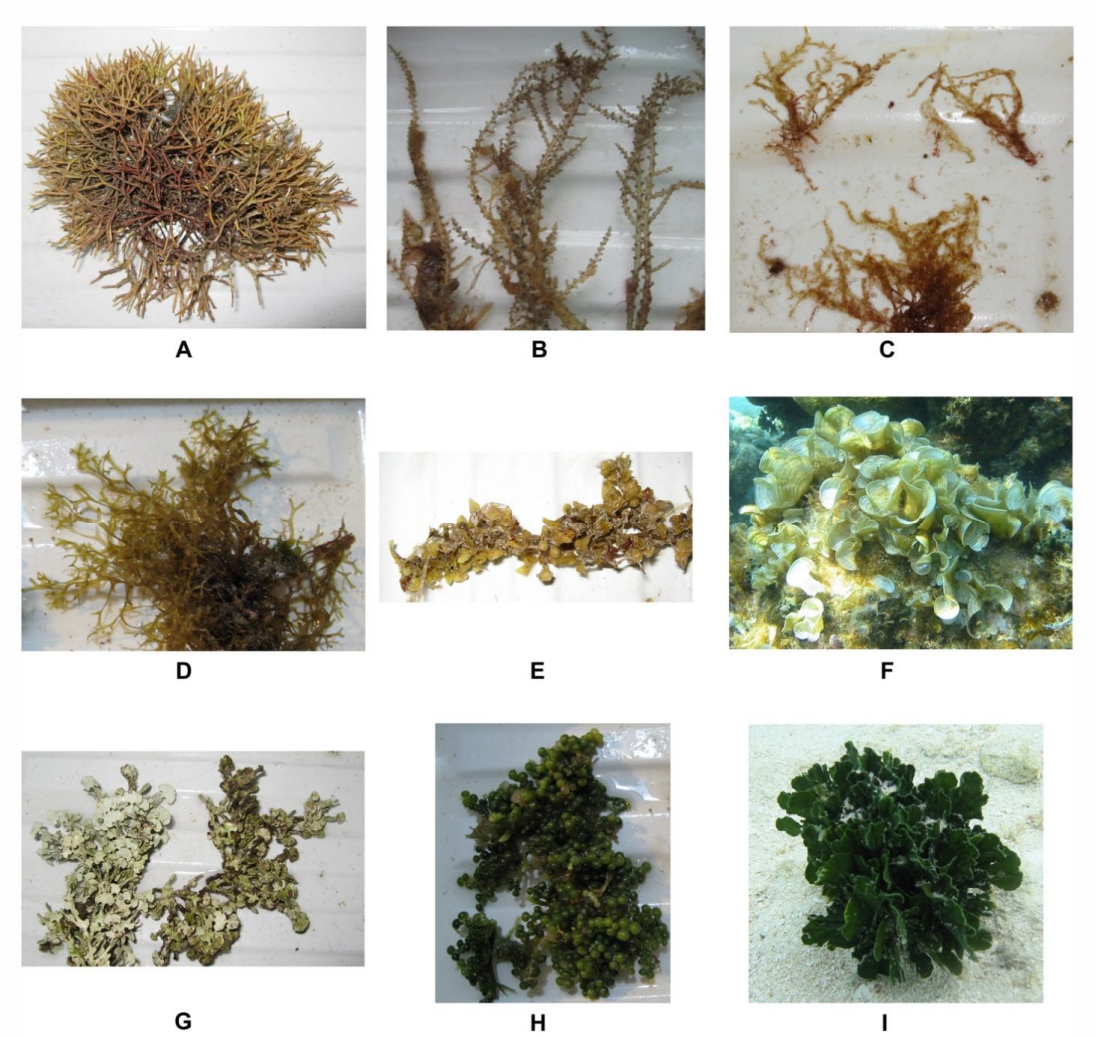


Figure 2. Shapes of common macroalgae of the Caribbean. **A.** *Amphiroa* sp. **B.** *Acanthophora* sp. **C.** *Heterosiphonia* sp. **D.** *Dictyota* sp. **E.** *Sargassum* sp. **F.** *Padina* sp. **G.** *Halimeda* sp. **H.** *Caulerpa* sp. **I.** *Udotea* sp. Credit: NOAA.

Other substrates

In addition to macrophytes, BHAB dinoflagellates have also been collected on a number of other benthic substrates. Certain substrates appear to be colonized preferentially by BHAB dinoflagellate species, such as specific taxa or morphotypes of macroalgae (Ballantine et al. 1985, Taylor and Gustavson 1985, Lobel et al. 1988). Other dinoflagellates tend to be generalists, associating with nearly any rigid substrate (Lawrence et al. 2000, Heredia-Tapia et al. 2002, Shah et al. 2010). After macrophytes, some of the more common substrates on which BHAB species have been collected include lithogenous materials such as coral debris, shells and rocks (Kohler and Kohler 1992, Grzebyk et al. 1994) or less commonly, detritus (Yasumoto et al. 1977a, Ballantine et al. 1985), floating/drifting algae (Ballantine et al. 1988, Rhodes et al. 2000) or artificial materials (Faust 1999). Regardless of which of these substrates are used, the sample is generally shaken and then sieved in a manner similar to the macrophyte method. The primary way in which these materials differ is in the units to which cell abundance is normalized and in the amount of contaminating sediment, detritus or other material present in the sample.

Suction sampling

Collection of benthic biota by suction sampling, otherwise known as vacuum sampling or hydraulic sampling, is another method that has been adapted to BHAB dinoflagellates. In short, dinoflagellate cells are collected from the surface of a substrate or from the sediment via vacuum pressure. Perhaps the simplest implementation of this method described in the literature is the use of a large syringe to collect suspended benthic matter (e.g., Porto et al. 2008). This method is limited to small amounts of sample, which may be sufficient for starting cultures, but not for assessing abundance. A larger sample size can be obtained by utilizing a vacuum pump (powered or manual), a collection bottle or flask, and a length of suction hose or tubing (Figs. 3A-C) (Kennelly and Underwood 1985, Atilla et al. 2003, Wahle et al. 2013). Some suction assemblies require a single diver/swimmer/wader (Boulton 1985), while others may require collection teams (True et al. 1968, Gulliksen and Deras 1975, Parsons et al. 2010) or ROVs (Robison et al. 2011). Some suction assemblies may be operated solely by surface personnel (Larsen 1974, Kikuchi et al. 2006). To date, the only instance of suction sampling of marine BHAB dinoflagellates was by Parsons et al. (2010), who employed a suction hose and flexible mesh skirt to quantitatively sample turf algae in the Hawaiian Islands.

Overall, some of the advantages of suction sampling are that the method can be adapted for all bottom types and sample collection is efficient in terms of time and effort for the size of the sample that can be collected (Kikuchi et al. 2006). The limitations are that the pumping equipment may be heavy and unwieldy, requiring two or more crewmembers to operate the larger suction systems. In addition, suction samples tend to be bulky, have great numbers of sediment particles and contaminating cells relative to the BHAB cells of interest. Furthermore, the turbulence of the suction system may cause cell damage and/or mortality, factors rendering

live cell collection potentially problematic. If live cells are desired, small hand vacuum systems are the preferred suction method.

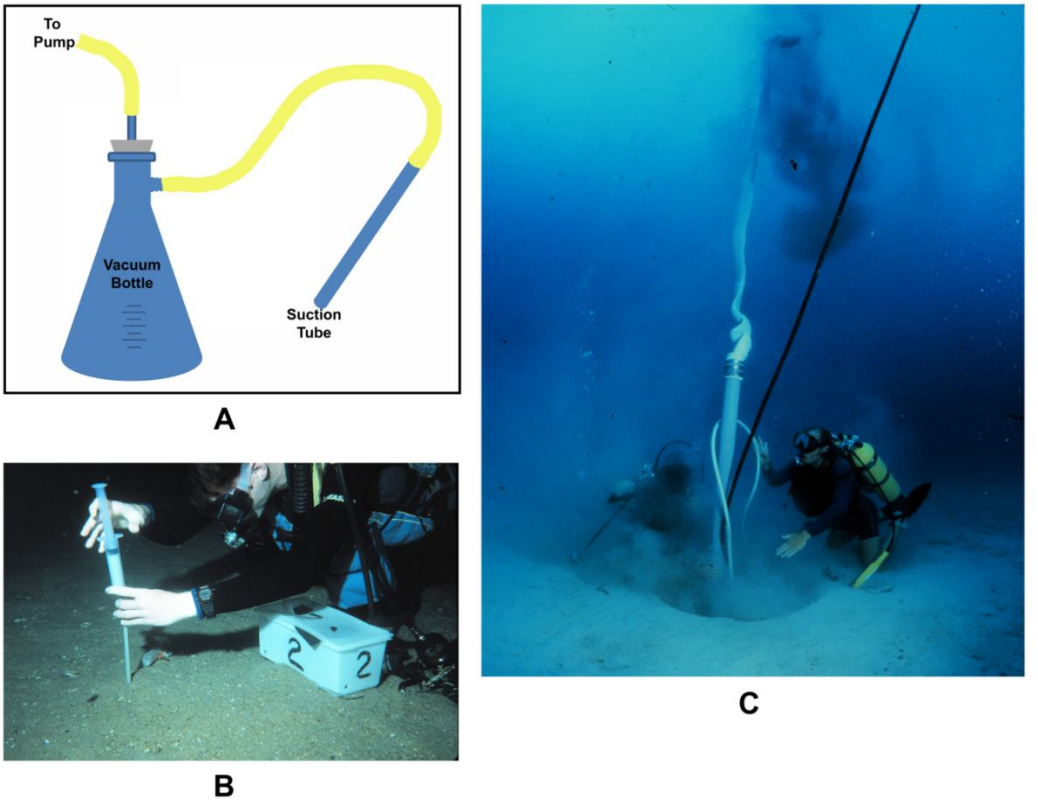


Figure 3. Suction sampling methods. **A.** Schematic of simple side-arm flask suction assembly for shallow water. **B.** Diver using a syringe for suction sampling. **C.** Diver-operated bottom dredge for large scale suction sampling. Credit: NOAA.

Part III: Sample separation and concentration

One of the most challenging aspects of sampling surface-associated dinoflagellates is physically separating the cells of interest from their substrates. In all but the cleanest samples, some method of separation is usually required before the benthic dinoflagellates can be quantified or isolated. No matter whether the collected substrate is macrophyte, coral, sand or other material, the resulting sample is a mixture of coarse to fine sediment, detritus, bits of macro- and microalgae as well as fauna. Because of the relative abundance of contaminating materials, which is typically 1-3 orders of magnitude greater than the cells of interest, contaminants are increasingly problematic as sample volume increases (Downing 1984). By far, the most commonly used method for separating benthic dinoflagellates from substrates is by shaking. For substrates which cannot be collected, such as large coral heads, rocks or pilings, some researchers have resorted to brushing or scraping to remove attached cells from the surface (Downing 1984, USGS 2002, Parsons et al. 2010). Others have used similar

mechanical methods to remove cells from macrophytes or lithogenous materials (Bagnis et al. 1980, Grzebyk et al. 1994, Lawrence et al. 2000, Mohammad-Noor et al. 2004, Okolodkov et al. 2007).

The level of agitation necessary to dislodge dinoflagellate cells from their substrates is rather subjective, prompting a wide array of descriptive terms in the literature detailing how much physical force is used (e.g., "gently", "moderately hard", "vigorously", etc.) and the duration that agitation is applied (seconds – minutes). In general, gentle shaking is sufficient to disperse cells which are only lightly associated with substrates, while more vigorous homogenization may be required for cells which are physically attached. The force and duration of homogenization may be tested easily by a stepwise increase in the amount/duration of force used, followed by visual inspection of the resulting suspension and/or examination of the substrate with microscopy. The degree of agitation also depends on whether the sample is live or preserved prior to processing. Live dinoflagellate cells subjected to physical stress during separation often become inactive, potentially losing one or both flagella. Some cells may even enter a temporary resting stage. Fortunately, most of the BHAB genera are thecate (*Gambierdiscus*, *Ostreopsis*, *Prorocentrum* and *Coolia*) and usually tolerate well the physical stresses incurred during sample processing. In particular, *Gambierdiscus* cells are quite resistant to damage during shaking, sieving or other modes of processing (Kibler et al. 2012). The sole athecate genus of BHAB dinoflagellates, *Amphidinium*, may be less tolerant of physical stresses, so more care is necessary during sample processing. Cell damage incurred during separation is an important consideration if live cells are desired for isolation and culture. Live cells collected for molecular analysis or toxin extraction need not be handled so carefully, so long as the cell contents are not lost before chemical extraction.

Size fractionation

Sieving

After the cells are separated from the substrate, further sorting is best achieved by size fractionation. The most common way is to use nested sieves. Sieves are available commercially through an array of scientific suppliers or can be user-built with nylon, metal or other appropriate mesh fabric. Perhaps the most common are metal sieves designed for sediment fractionation or custom sieves made from nylon or fiberglass mesh glued to short pieces of plastic pipe (Fig. 4). Generally, a cell sample is poured through multiple sieves with decreasing pore sizes to fractionate particles. Sieves with a relatively large pore size are employed to remove bigger particles from the sample, and smaller pore size sieves are used to collect the cells of interest and/or remove smaller particulates. A stream of filtered seawater is typically used to rinse particles though the mesh and to backwash or transfer cell samples into a container. One of the limitations of sieving is the amount of sample that can be processed. This will depend on the diameter and pore size of the sieve(s), the size range and quantity of particulates in the sample, the characteristics of the cells of interest and the desired volume of the processed sample. Larger samples or those with many small particles are more difficult to

size fractionate, requiring more seawater rinses during sieving. The size fractionation method can be rendered quantitative provided the volume of seawater is recorded. However, the sample volume should be measured *before* the sieving step to account for the dilution that occurs during the fractionation process. Once the cell concentration is determined, it can be normalized to macrophyte mass, surface area or sediment volume-based units.

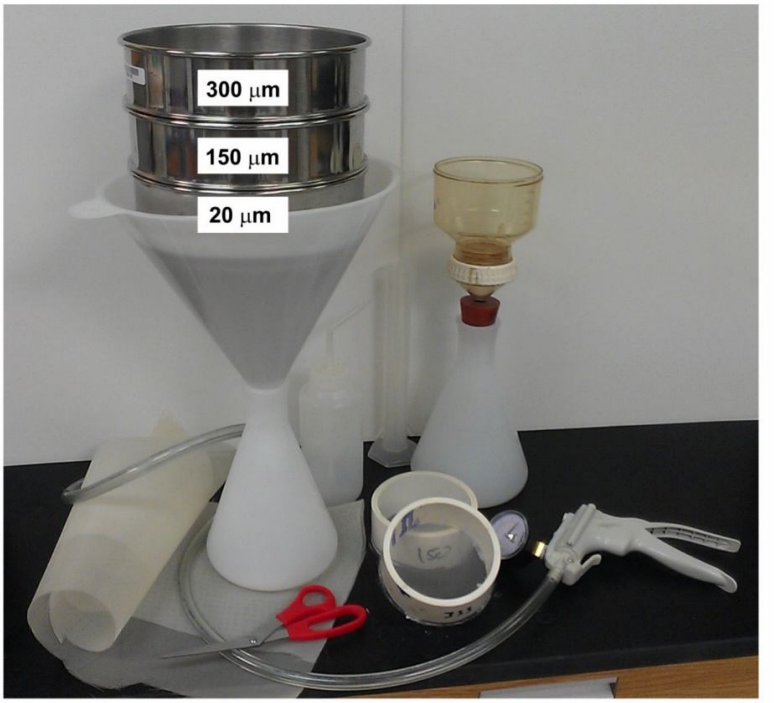


Figure 4. Sieves and filtration labware for size fractionating BHAB dinoflagellates. Credit: NOAA.

After sieving, the particulates remaining in the sieved sample have a specific size range (e.g., <100 µm, >20 µm, 30-70 µm) depending on the sieves used. Assuming microscopy will be used to examine the processed sample, the sample should span the narrowest possible size range to limit contaminating cells and other particulates. For surface-associated dinoflagellates, the genera *Gambierdiscus* (39-115 µm diameter) and *Ostreopsis* (18-166 µm diameter), include the largest of the BHAB species, most having an effective diameter of >100 µm (Table 2). However, some species of *Ostreopsis* may be as small as 18 µm. Similarly, some *Prorocentrum* species reach a relatively large size (70-80 µm), while others are among the smallest BHAB cells (~13 µm). *Coolia* and *Amphidinium* cells tend to be of smaller size, ranging between 20 and 65 µm (Table 2).

Table 2. Cell size ranges for benthic harmful algal bloom (BHAB) dinoflagellate genera.

Genus	Effective diameter (µm)	Reference
Gambierdiscus	39 – 115	Litaker et al. 2009 Fraga et al. 2011 Fraga and Rodríguez 2014 Nishimura et al. 2014 Kretzschmar et al. 2017
Ostreopsis	18 – 166	Faust 1999 Accoroni et al. 2012
Prorocentrum	13 – 80	Lu & Goebel 2001 Cohen-Fernández et al. 2006
Coolia	21 – 65	Tolomio and Cavalo 1985 Faust 1995
Amphidinium	20 – 50	Murray et al. 2004 Dolapsakis and Economou-Amilli 2009 Gárate-Lizárraga 2012

The wide cell size range among the BHAB species may complicate size fractionation, especially if all of the BHAB genera are of interest. Because microscopy of sieved samples must be considered, the particular size range that is employed should take into account the species of interest, the amount of time and effort necessary for sample processing as well as the objectives of the particular study. For studies targeting all BHAB cells, some authors have limited size fractionating to include only coarse sieves to remove sand and large particles (e.g., 500 µm) (Kim et al. 2011). Although no BHAB cells are (theoretically) lost by this method, cells in the resulting sample may be difficult to recognize among contaminating particles. A narrower size range is therefore more commonly employed (e.g., 25-75 µm) (Delgado et al. 2006) to eliminate sand, zooplankton and large detritus as well as very small contaminating cells and particulate matter. The latter approach may yield samples with fewer contaminants, but both very large and very small BHAB cells may be lost during processing. The use of a wider size range, such as that employed by Grzebyk et al. (1994) (20-140 µm), or the retention of more than one size fraction (e.g., 20-50 µm and 50-140 µm) may be the best solution for most BHAB species.

Preserved samples

Chemical preservation is typically used when samples cannot be processed immediately, if samples are going to be archived for subsequent analysis or if the separation process may cause cell damage or mortality. The use of iodine or aldehyde fixatives has been incorporated successfully into sampling protocols of many studies seeking to quantify cell abundance (e.g., Cohu et al. 2011, Reguera et al. 2011, Asnaghi et al. 2012). Material for cell enumeration is generally fixed before, during or immediately after cells are separated from their respective substrates (e.g., Kim et al. 2011, Richlen and Lobel 2011). The cross-linking of proteins that

occurs in cells during fixation makes cells less susceptible to physical damage during handling, although substantial changes in cell size may occur during fixation (Fox et al. 1985, Zarauz and Irigoien 2008, Nowacek and Klernan 2010). Furthermore, some BHAB species may be poorly preserved with particular fixatives, so prior knowledge of the chemical effects of the fixative used on the cells of interest is recommended. The most common fixatives used for BHAB research are Lugol's iodine solution (Steedman 1976, Throndsen 1978), as well as formaldehyde and glutaraldehyde (e.g., Aligizaki and Nikolaidis 2006, Faust et al. 2008, Kim et al. 2011). The benefits and detriments of various fixatives are discussed elsewhere (Kimor 1976, Throndsen 1978, Fox et al. 1985, Auinger et al. 2008, Nowacek and Klernan 2010). See Chapter VI.

Sonication

Another method for separation of BHAB cells from particulates and contaminating material is sonication. Sonication refers to the use of high frequency vibration to agitate and disperse particles within the sample. There are two types of instruments available for this purpose—sonication probes and sonication baths (Fig. 5). A sonication probe has a metal tip which is inserted into a sample container from above while a sonication bath is a specialized water bath where the vibration is introduced from below into the water. Sonication is generally employed to break up clumps of sediment and detritus just prior to size fractionation to facilitate removal of contaminating particles. The method has been used to separate bacteria, dinoflagellate cysts and other structurally resistant materials from fine sediment particles (Legendre 1993, Kendall et al. 2003, Kennison et al. 2003, Kostylev 2012). Less commonly, sonication has been employed to separate live algal cells from sediment (Lessios 1996). For benthic dinoflagellates, the method has been employed on both live samples (Marasigan et al. 2001, Vila et al. 2001) and preserved material (Faust 1995, Bez 2004, Bataineh et al. 2006, Faust et al. 2008, Totti et al. 2010). Great care must be taken to ensure the cells of interest are not destroyed with the contaminating material regardless of the sonication method. High energy sonication has been shown to cause algal cell mortality even at short exposures (Elphick 2008). Therefore, the sonication energy should be high enough to disperse clumps of particulate matter without damaging the BHAB dinoflagellate cells of interest. The amount of energy applied, and the duration of sonication are best determined by trial and error with a few preliminary samples.

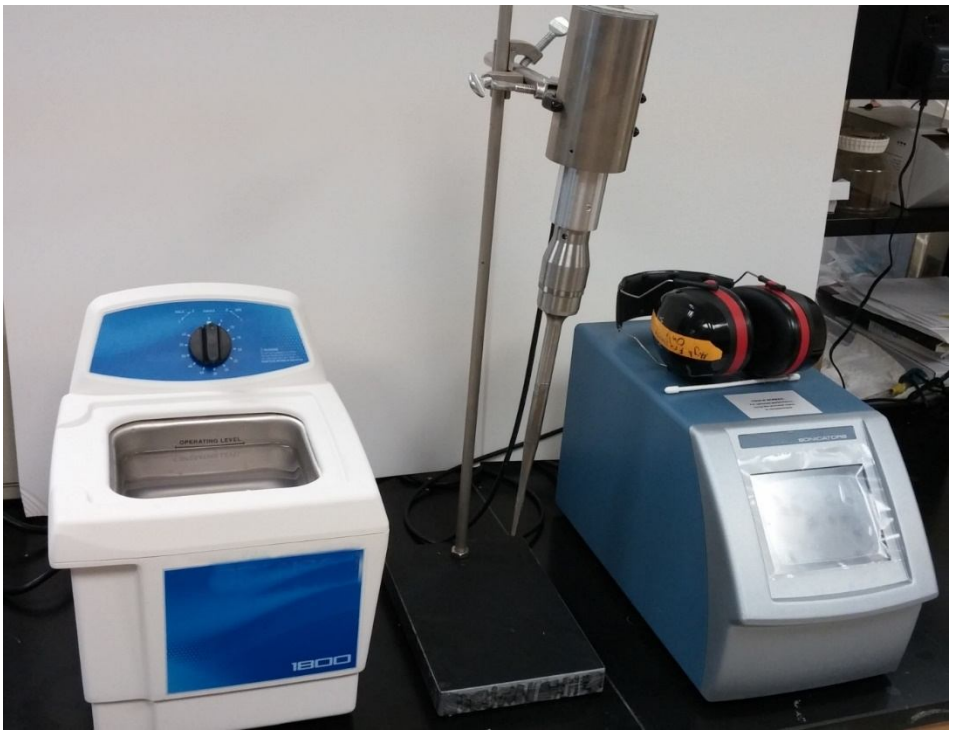


Figure 5. Sonicating bath (left) and sonicating probe (right) used for disaggregation of sediment or other samples. Credit: NOAA.

Filtration

Another method available to size fractionate BHAB cell samples is filtration, here defined as the use of water or vacuum pressure to force the sample through a single-use filter. Because of the physical stresses cells are exposed to during filtration, the method is often used to concentrate fixed cell samples, although some researchers have employed it before fixation as well (Clements et al. 2010, Tester et al. 2014). Membrane filters are most commonly used for size fractionation because their pore size is better defined than paper or glass fiber filters. Unlike sieves, a separate filter membrane is used for each sample, eliminating the cleaning and back washing steps required when using sieves. However, the filter base, funnel and filter support must be cleaned to prevent carryover (see below) unless disposable funnels are utilized.

To concentrate cells, the sample may be vacuumed onto the filter membrane and then rinsed off the filter into another container. As an alternative, some researchers have used a transparent or semi-transparent filter membrane to concentrate a sample and then used microscopy to examine the membrane itself (Brown et al. 2004). For this method, the filter membrane is typically mounted on a microscope slide with immersion oil and the cells are visually counted. The filtration method has been used successfully with cell stains or epifluorescent microscopy for identification and enumeration of microalgae in plankton samples for many years (Brown

et al. 2004, Cabral and Murta 2004). With little difficulty, the method may be adapted to concentrate BHAB dinoflagellate samples.

An advantage of vacuum filtration is that the vacuum pressure allows a higher degree of concentration than is generally possible during sieving (i.e., gravity filtration). This process can aid in enumeration of cells normally present at low densities, but may over-concentrate those present at high densities. A disadvantage to the filtration method is that cells and other contaminating particulates may become compacted on the filter membrane, potentially making cell identification and enumeration more difficult (Dethier and Schoch 2005). For this reason, it is generally beneficial to pre-fractionate samples with a sieve to reduce contaminating particles before the filtration step. More details about the filtration method are provided in Dethier and Schoch (2005) and Ardisson and Bourget (1992).

Sample carry-over

Because size fractionation procedures typically entail the use of labware, such as filter funnels and supports, graduated cylinders and sieves to process multiple samples, cross-contamination among samples is a potential problem. Contamination arises when cells or their contents are carried over into subsequent samples. This is seldom a problem for studies using microscopy to visually identify and count BHAB cells of interest because very few whole cells are transferred between samples. However, such low-level contamination is a very real concern when sensitive PCR assays are used.

Methods such as PCR represent powerful tools that are able to detect only a few molecules of target DNA from a complex mixture of other DNAs. As a result, a small amount of DNA carry-over from one sample can cause “false positive” results in subsequent samples (Jokiel et al. 2005). Cross-contamination among samples is particularly problematic for assays targeting RNA genes, of which there may be hundreds of copies per cell (Hill and Wilkinson 2004, Auinger et al. 2008). Adhesion of DNA/RNA can occur on many surfaces used to size fractionate samples, including the bottom and sides of graduated cylinders and beakers, the inner surfaces of filter funnels and supports, and the mesh of sieves. This contaminating nucleic acid may be surprisingly resistant to routine cleaning. For example, Hill and Wilkinson (2004) found that washing and autoclave sterilization are inadequate to eliminate DNA contamination in equipment between samples.

Instead, a series of precautionary steps should be included in BHAB sampling and processing protocols. One method for preventing molecular carryover is to soak fractionation labware in a bath of 10% bleach for 5-10 minutes after all particulate materials have been rinsed away. The bleach treatment should include all sieves, funnels and labware that are utilized. Any mesh sieves should be backwashed thoroughly before soaking them in bleach. Because metal sieves may corrode from the caustic bleach solution, sodium hypochlorite residue should be rinsed away with tap water followed by deionized water to avoid degradation of sieve frames, mesh and the associated sealant. Another method to eliminate DNA carryover is to treat

fractionating materials with commercial products such as DNAZap™ (Life Technologies Corp., Carlsbad, California, USA), which rapidly degrades nucleic acids on contacted surfaces. Alternatively, disposable funnels, forceps and pieces of sieve material may be employed during fractionation to increase sample throughput. More specific information regarding the prevention of molecular contamination/carryover is given elsewhere (Litaker et al. 2003, Auinger et al. 2008, Battocchi et al. 2010, Vandersea et al. 2012).

The settling method

Settling chambers are commonly used for concentrating, examining and enumerating BHAB dinoflagellates from substrate samples. Otherwise known as the sedimentation method, settling has been adapted from the method detailed by Utermőhl (1931, 1958) for phytoplankton samples. In short, a defined volume of a preserved cell sample is placed in a chamber with a flat, transparent bottom, allowed to settle, and the cells of interest are counted via inverted microscopy. Ideally, replicate aliquots are counted, the cell concentration is calculated as the average of these. Because BHAB species can occur over a wide range of abundances, an appropriate settling volume should be selected. Some of the most common settling chambers are manufactured by Hydro-Bios (Germany, http://www.hydrobios.de). These are made of high density plastic with a thin glass bottom and are available in 5, 10, 25, 50 and 100 mL volumes (Barnes 2010). The appropriate chamber volume depends largely upon the abundance of the cells of interest and whether the whole chamber will be counted or only portions thereof (Jauzein et al. 2018). Various subsampling methods for determining cell concentrations have been devised, where portions of the chamber are used as an estimate for the entire volume (Crabbe 2008, for details, see Gimenez et al. 2010, Ayotte et al. 2011).

The settling chamber method is ideal for samples where the BHAB cells of interest are present at abundances ≥10 cells mL^{-1} of sample. At this concentration, a 100 mL settling chamber would contain ~1,000 cells, providing sufficient precision among replicate counts for most field studies (Govindasamy and Anantharaj 2012). Unfortunately, the abundance of BHAB dinoflagellates is often far less than 10 cells mL^{-1} of sample. As a result, it may be necessary to settle as much as several liters of sample to achieve the desired density for cell counting. These volumes are well beyond the normal sample volume for substrate-associated dinoflagellates. For samples >100 mL, an alternative is to settle the sample in a large graduated cylinder, remove the supernatant by aspiration or suction after an appropriate settling time, and then transfer the concentrated sample to a settling chamber for enumeration. The major sources of error with this method involve the accuracy of the cylinder, adhesion of cells to the cylinder walls and cells lost during the aspiration and transfer of the sample.

Another consideration for concentrating samples is the time required for all cells to settle to the bottom of the chamber. Giménez et al. (2010) indicated 24 hours as an adequate settling time for large chambers up to 100 mL. Because settling chambers vary in size, Edgar and Klumpp (2003) recommended that a sedimentation time of 4 hours per centimeter chamber height was sufficient for even the smallest cells. For large BHAB dinoflagellates such as

Gambierdiscus (>40 µm), settling times for iodine-fixed cells tend to be rapid, 30-60 min are generally sufficient for 5-10 mL settling volumes. For smaller BHAB cells (20-30 µm), settling times of 1-2 h are more appropriate. Settling time also depends upon the chemical fixative that is used. The advantage of Lugol's iodine solution is that it increases the weight of the organisms, thereby reducing sedimentation time (Barnes 2010). Aldehyde-fixed samples may require longer settling times. Centrifugation may also be used in conjunction with the settling method to decrease settling times. Most commonly, centrifugation has been used to concentrate cells for enumeration, microscopy, molecular analysis or toxin extraction (Buckland et al. 1993, Rhodes et al. 2000, Penna et al. 2005, Ciminiello et al. 2006, Hoppenrath and Leander 2008, Amorim et al. 2010, Bruckner and Renaud 2012). Also see Chapter VI for further information on settling methods.

Non-quantitative methods

Other, non-quantitative methods may be employed to separate cells from substrates and contaminating material if cell abundance is not of interest. For instance, some researchers have used cell behavior to aid separation. Ballantine et al. (1988) noted that after macrophyte samples were shaken, *Ostreopsis* cells tended to form clumps or strands of cells when incubated in a shallow tray under a light source for a few hours. Aspiration was then used to collect the clumps of cells for subsequent microscopy and toxin extraction. A similar method has been employed to concentrate BHAB cells in sediment and detritus samples for SEM (M.A. Faust, personal communication). If a small number of cells are desired, an effective but more laborious method for concentration is micropipetting, where a thinly drawn pipette is used to manually separate dinoflagellate cells for more detailed examination, live culture isolation, molecular analysis or SEM (Faust 1995, 2008, Litaker et al. 2009).

A more elegant method employed to separate live substrate-associated dinoflagellates is the melting seawater method of Uhlig (1964), which has long been employed to separate interstitial biota from sediment samples. Briefly, seawater ice is placed atop a short column of sediment suspended over a sieve or filter cloth. A Petri dish or similar container of filtered seawater is then placed under the column. The downward-moving thermosaline gradient produced by the melting ice causes ciliates, flagellates and other motile organisms to move through the filter mesh into the dish, where they are then collected. Although Uhlig (1964) described the method as nearly 100% efficient at extracting motile biota, subsequent comparisons (e.g., Martens 1984) have shown the results depend greatly on the organisms of interest and their degree of motility, as well as the amount of sediment and the geometry of the extraction column. The primary use of this method is for qualitative studies on interstitial fauna, such as descriptions of new benthic dinoflagellates species (e.g., Hoppenrath and Leander 2008, Yamaguchi et al. 2011, Hoppenrath et al. 2014).

Part IV: Artificial substrates

As an alternative to collecting macrophytes some researchers have employed artificial substrates to collect BHAB dinoflagellates (Table 3). Briefly, an artificial substrate is placed at the study site, allowed to incubate for a defined time period and then retrieved and processed like natural substrate samples. This approach is based on the observation that benthic HAB species have been observed in the water column and are able to colonize new substrates. Therefore, the colonization of a new surface is a function of the population size within the vicinity of the artificial substrates. Field tests have repeatedly shown BHAB cells recruit to artificial substrates placed near the bottom (Fig. 6A). This is the case for all suitable habitats, including those devoid of macrophytes, turf algae or other natural surfaces (i.e., bare sand/mud flats) (Tester et al. 2014).

The use of artificial substrates for collection of surface-associated biota is commonly seen in freshwater studies. Some of the substrates used in both freshwater and marine environments include baskets or bags of gravel (Shieh and Yang 1999, Czerniawska-Kusza 2004, Holmes et al. 2005), bricks (Fairchild and Holomuzki 2005), glass slides (Tippett 1970, Tonetto 2010, Tonetto et al. 2012) and panels or plates made of a variety of materials (Meier et al. 1979, Canton and Chadwick 1983, Hill and Matter 1991, Letovsky et al. 2012) (Table 3). Sampling devices equipped with artificial surfaces are also commercially available (e.g., Periphyton Sampler, Wildlife Supply Co., Yulee, Florida, USA, http://www.wildco.com; Hester-Dendy Sampler; Envco Environmental Equipment Suppliers, Auckland, New Zealand, http://www.envcoglobal.com) (Figs. 6B-C). These artificial materials have most frequently been utilized to collect periphyton, aquatic insects and benthic invertebrates (Table 3). The use of artificial substrates has been adopted as a standard protocol in several aquatic monitoring programs (e.g., Klemm et al. 1990, Holmes et al. 2005).

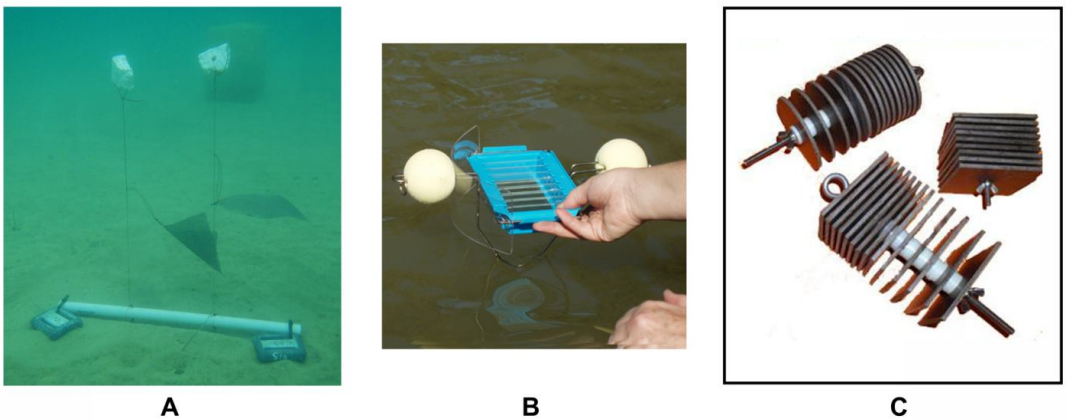


Figure 6. Artificial substrates used to collect surface-associated biota. **A.** Rectangles of fiberglass window screen used to collect BHAB dinoflagellates. Credit: NOAA. **B.** Periphyton sampler with glass microscope slides. Credit: Wildlife Supply Co., Yulee, Florida, www.wildco.com **C.** Hester-Dendy sampler for aquatic invertebrates. Credit: Envco Environmental Equipment Suppliers, Australia, www.envcoglobal.com.

Table 3. Artificial Substrates and aquatic biota they were used to collect.

Substrate	Organism	Environment	Incubation time	Reference
Glass slides	Periphyton	Freshwater	5-91 d	Tonetto 2010 Tonetto et al. 2012
Bricks	Insect larvae	Freshwater	?	Fairchild and Holomuzki 2005
Gravel-filled baskets	Benthic macroinvertebrates	Freshwater	4-6 wk	Holmes et al. 2005
Multi-plate collector	Aquatic insects	Freshwater	5 wk	Canton and Chadwick 1983
Gravel-filled bags	Macroinvertebrates	Freshwater	~1 mo	Czerniawska-Kusza 2004
Multi-plate collector	Macroinvertebrates	Freshwater	28 d	Hill and Matter 1991
Multi-plate collector	Macroinvertebrates	Freshwater	10 d	Letovsky et al. 2012
Multi-plate collector	Macroinvertebrates	Freshwater	18-39 d	Meier et al. 1979
Gravel-filled baskets	Aquatic insects	Freshwater	3-42 d	Shieh and Yang 1999
Glass slides	Microalgae	Freshwater	Biweekly	Tippett 1970
Plastic mesh pad Bottle brushes Multi-plate collector	Estuarine meiofauna	Estuarine	24 h	Atilla and Fleeger 2000
Ceramic tiles	Calcareous invertebrates	Marine	4-8 mo	Holmes et al. 1997
Plastic tape strips	Benthic dinoflagellates	Marine	2+ mo	Caire et al. 1985
Fiberglass disks	Fouling biota	Marine	2-16 d	Caron and Sieburth 1981
Aluminum plates	Fouling biota	Marine	~1 mo	da Fonsêca-Genevois et al. 2006
Rope fibers Plastic strips	Epifaunal invertebrates	Marine	6 wk	Edgar and Klumpp 2003
Plastic plates	Mangrove root biota	Marine	2 wk-9 mo	Elliot et al. 2012
Metal disks	Bacteria	Marine	1-12 d	Guezennec et al. 1998
Plastic mesh ovoids	Mussels	Marine	1-2 mo	Howieson 2006
Plastic panels	Bacteria and diatoms	Estuarine	3-22 wk	Hudon and Bourget 1981
Glass disks	Periphyton	Marine	2 wk	Schmitt-Jansen and Altenburger 2005
Polyester grass	Epiphytic microalgae	Marine	Monthly	Tanaka et al. 1984
Polypropylene grass	Epiflora and fauna	Marine	12 h-16 d	Virnstein and Curran 1986
Glass slides	Epiphytic ciliates	Marine	10 d	Xu et al. 2009
Ceramic plates Coral plates	Epifauna	Marine	6 mo	Dulvy et al. 2002
Polyester grass	Microgastropods	Marine	6-27 d	Olabarria 2002
Plastic seagrass	Meiofauna	Marine	2-21 d	De Troch et al. 2005
Plastic seagrass	Meiofauna	Marine	2-21 d	De Troch et al. 2005
Test tube brushes Plastic plates	Benthic dinoflagellates	Marine	?	Bomber and Aikman 1989
Fiberglass screen	BHAB dinoflagellates	Marine	24 h	Tan et al. 2013 Tester et al. 2014 Jauzein et al. 2016, 2018

In marine studies, the most common artificial substrates have been pieces of rope fiber, ceramic, metal, plastic or glass, sometimes fashioned to approximate the shapes of seagrasses or algae (Table 3). Surface associated biota that have been collected in marine systems include bacteria (Hudon and Bourget 1981, Guezennec et al. 1998), epiphytic microalgae (Tanaka et al. 1984, Schmitt-Jansen and Altenburger 2005), micro- and macroinvertebrates (Holmes et al. 1997, Dulvy et al. 2002, Edgar and Klumpp 2003), mangrove prop root biota (Elliot et al. 2012), as well as various fouling organisms (Caron and Sieburth 1981, da Fonsêca-Genevois et al. 2006). There have been very few studies employing artificial materials for BHAB research. Caire et al. (1985) utilized a series of fabric strips suspended in the water column to monitor the *Gambierdiscus* population at a French Polynesian atoll. Similarly, artificial materials (test tube brushes, plastic plates) were used to compare the abundance of *Prorocentrum lima* on substrates with different surface areas in the Florida Keys, USA (see Bomber and Aikman 1989). More recently, an artificial substrate method for assessing BHAB dinoflagellate abundance using pieces of window screen has been tested in a range of tropical and subtropical marine environments (Kibler et al. 2010, GEOHAB 2012, Tester et al. 2014, Jauzein et al. 2016, 2018). Ishikawa et al. (2011) used a similar substrate to collect BHAB dinoflagellates in Japanese coastal waters. A recent initiative by the Intergovernmental Oceanographic Commission – Scientific Committee on Oceanic Research (IOC-SCOR) Global Ecology and Oceanography of Harmful Algal Blooms (GEOHAB) program funded by the Yeosu Project resulted in a workshop featuring the use of the screen substrates as a monitoring method for *Gambierdiscus* and other potentially toxic benthic dinoflagellates (GEOHAB Workshop 2012). Jauzein et al. (2016) have recently optimized the window screen artificial substrate method for use with *Ostreopsis* cf. *ovata* blooms. The method was also adopted by the European Union-funded M3-HABs monitoring group, a consortium of universities, research institutes, environmental agencies and technological enterprises based along Mediterranean coasts (http://m3-habs.net/category/guidelines-protocols/).

Considerations

There has been uncertainty within the BHAB research community concerning the use of artificial substrates stemming from a lack of data that directly compare BHAB dinoflagellate abundance on natural and artificial materials. A critical question has arisen: Do artificial substrates function like natural substrates? To address this question a recent study by Tester et al. (2014) has compared the artificial substrate method with the macrophyte method in a variety of environments in Belize, Central America and Malaysia (GEOHAB Workshop 2012). Some of the results from side-by-side comparisons are given in Fig. 7, which shows the abundances of *Gambierdiscus*, *Ostreopsis* and *Prorocentrum* species associated with an artificial substrate (fiberglass screen) and a range of different macrophytes. Despite variability among replicates and very different quantitative scales, there was an obvious association between abundances on screens (cells 100 cm^{-2}) and abundance on macrophytes (cells g^{-1}). This relationship is clear when average cell abundance on screens at each site is viewed as a function of abundance on macrophytes at the same site ($r^2 = 0.99$, $p<0.001$) (Fig. 8).

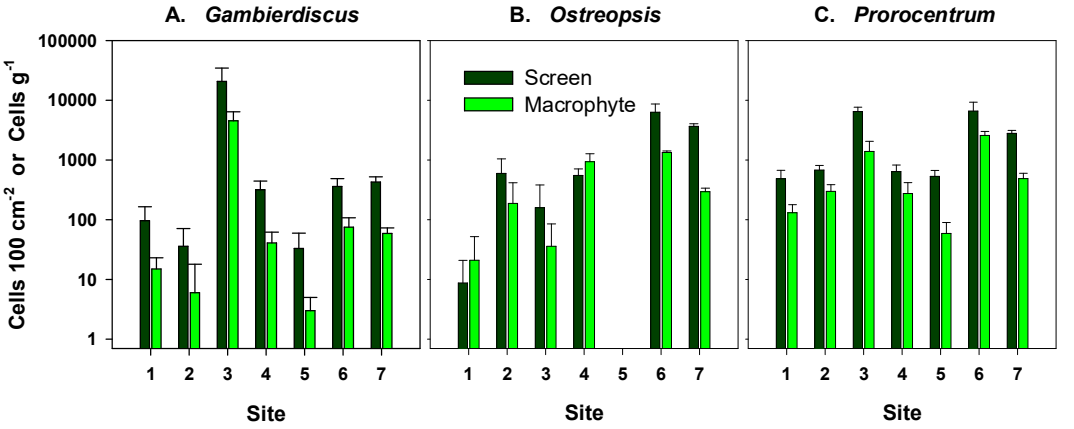


Figure 7. Comparison of abundances of **A.** *Gambierdiscus,* **B.** *Ostreopsis,* and **C.** *Prorocentrum* cells associated with an artificial substrate (fiberglass window screen, cells 100 cm^{-2}) and a variety of macrophyte species (cells g^{-1}) at seven sites in the Central Lagoon of Belize (sites 1-7), Central America (May 2009); from Tester et al. (2014, Vol. 39: p. 15, fig. 6).

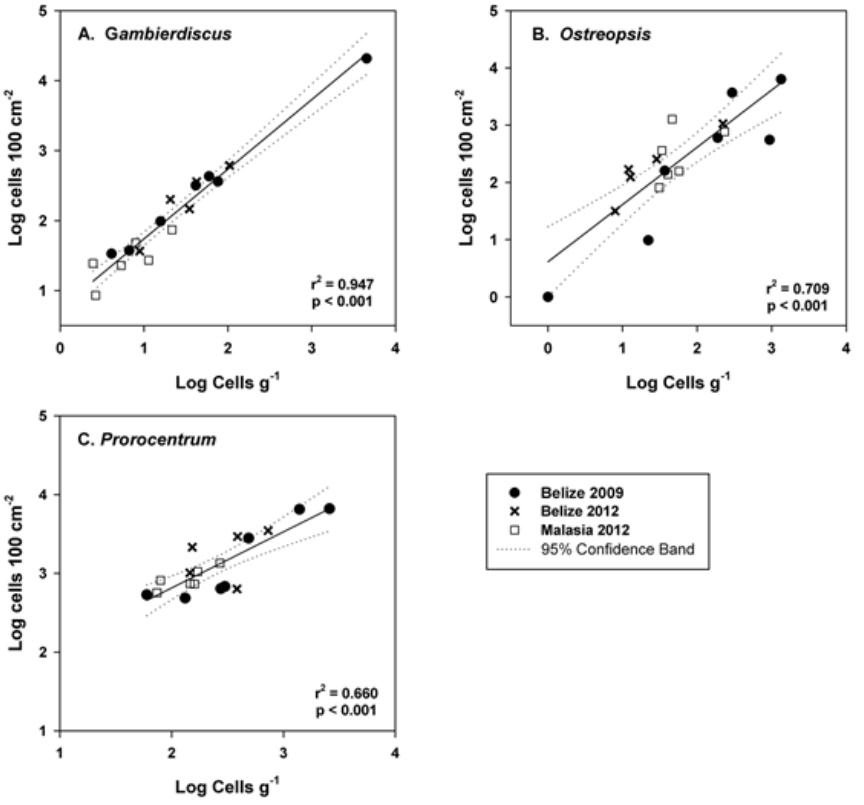


Figure 8. Relationship between average abundance of **A.** *Gambierdiscus,* **B.** *Ostreopsis,* and **C.** *Prorocentrum* cells associated with replicate screens (cells 100 cm^{-2}) and algae (cells g^{-1}) samples collected at sites in Belize (May 2009, January 2012) and Malaysia (May 2012). Linear regression (solid lines) and 95% confidence intervals (dotted lines) are shown with regression coefficients and associated p-values. Data were log-transformed prior to regression analysis and have been plotted on a linear scale; from Tester et al. (2014, Vol. 39: p. 17, fig. 7; p. 18, fig. 8; p. 19, fig. 9).

Little is currently known about the rate at which new substrates are colonized by surface-associated dinoflagellates. Biofouling studies have shown colonization of new surfaces involves a progression beginning with rapid formation of an organic microgel/exopolymer layer and colonization by marine bacteria and picoalgae within minutes of incubation, followed by diatoms and other microalgae (Hudon and Bourget 1981, Mitbavkar et al. 2012). Colonization by meiofauna, such as nematodes, bivalve larvae and harpacticoid copepods follows soon after within 1-2 days (da Fonsêca-Genevois et al. 2006). Association with substrates by BHAB dinoflagellates follows a similar pattern. Tester et al. (2014) demonstrated recruitment of *Gambierdiscus*, *Ostreopsis* and *Prorocentrum* cells to fiberglass screens occurred within 12-18 h of incubation and BHAB abundances reached equilibria with the surrounding dinoflagellate population within 24 h. The authors pointed out that BHAB cells immigrating to artificial substrates likely originate from the surrounding near-bottom water column, from the bottom itself, or from nearby natural substrates. In contrast, Caire et al. (1985) found that *Gambierdiscus* cells at a Pacific atoll did not appear on artificial substrates (fabric strips) until after 2-3 months of incubation. Similarly, an incubation period of approximately one month has been adopted for artificial substrates (tiles) in a current study in the greater Caribbean region (CiguaHAB 2016). Given the paucity of data concerning the rate of association, immigration of BHAB cells to both artificial and natural substrates needs to be examined more closely.

Characteristics potentially governing colonization by benthic dinoflagellates include chemical composition of the substrate, surface texture (roughness, porosity, etc.), available surface area and a range of site-specific characteristics such as flow or wave energy. Among these, available surface area has been examined among macrophytes, where filamentous algae having high surface area to volume ratios sometimes exhibited higher dinoflagellate abundances (Bomber 1985, Taylor 1985, Taylor and Gustavson 1985). It follows that artificial materials having relatively high surface areas (brushes, frayed rope, woven mesh or cloth) may be expected to host higher BHAB abundances as well. Currently the only study examining colonization of artificial substrates of different surface areas by dinoflagellates was a preliminary study to compare the abundance of the benthic dinoflagellate *Prorocentrum lima* on test tube brushes and plastic plates (see Bomber and Aikman 1989). The results indicated an order of magnitude higher abundance on the brushes. Similar studies of aquatic meiofauna and periphyton showed artificial materials with high surface areas (bottle brushes, plastic mesh pads, rope fiber tufts) were colonized more rapidly than less complex structures, but with a similar assemblage of biota (Atilla and Fleeger 2000, Edgar and Klumpp 2003, Richard et al. 2009).

Advantages and disadvantages

Artificial substrates offer numerous advantages over macrophytes or other natural substrates for assessment of surface-associated dinoflagellates. These advantages simplify some of the most problematic aspects of natural substrate sampling. First, the artificial substrate method is nondestructive, thus preserving environmentally sensitive habitats such as coral reef, seagrass

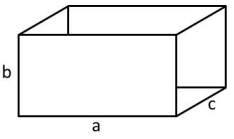
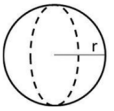
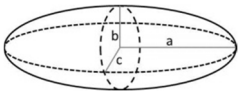
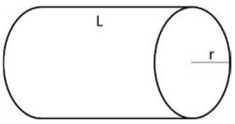
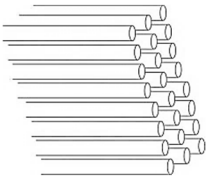
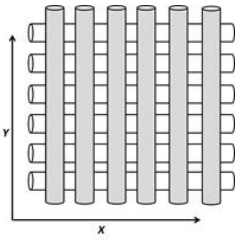
or mangrove environments. Because macrophytes are no longer required for BHAB species assessment, the method also eliminates purported dinoflagellate-macroalgae preference effects as well as grazing by fish or other fauna. Furthermore, algal palatability/defense factors that may complicate CFP toxin transfer pathways are not applicable to artificial substrates (see Cruz-Rivera and Villareal 2006). The artificial substrate method also eliminates difficulties with substrate availability.

Other benefits of the artificial substrate method are more practical in nature. For instance, artificial substrates are generally easy to deploy and retrieve with no complicated equipment. The materials used are inexpensive (e.g., fiberglass window screen) and sampling units can be replicated easily and randomized, allowing field studies with sophisticated sampling designs to be created. This benefit is particularly useful given the patchy distribution of natural macrophytes, which often limits spatial/temporal comparisons with natural substrates. For instance, replicate samples of the same macroalgal species can often be difficult to find within the same location due to grazing by herbivorous fauna, spatial heterogeneity in macrophyte growth condition and a number of other factors governing substrate distribution. Replicates of artificial substrate can be placed in nearly any environment and at a variety of spatial or temporal scales. From the standpoint of microscopy or isolation and culture of BHAB dinoflagellate cells, artificial substrate samples tend to be much cleaner than those from natural substrates, with fewer contaminating biota and less sediment. This characteristic is also beneficial when cell abundance is quantified, making BHAB cells easier to identify and enumerate via microscopy.

The most significant advantage of the artificial substrate method concerns normalization of cell abundances and inter-comparison of different substrates. As detailed in Part II, it has long been recognized that dinoflagellate cell densities are best normalized to substrate surface area (cells cm^{-2}, cells 100 cm^{-2}), rather than mass (cells g^{-1}) or volume (cells cm^3). Such normalization enables direct comparison of cell abundances among a range of surfaces including different species and shapes of macroalgae, seagrasses, coral fragments and other materials. The surface area of artificial substrates, particularly those having a regular geometric shape, is generally much easier to estimate than are natural materials such as leafy or filamentous macroalgae. Examples of such flattened materials include ceramic tiles and plastic sheets or strips. By adapting simple geometric formulae (Table 4), the surface area of more complex materials such as plastic filaments or screen mesh can also be approximated. For instance, the abundance of *Gambierdiscus* cells in a seagrass bed (cells cm^{-2}) can be directly compared to the abundances in a patch reef (cells cm^{-2}) by placement of artificial substrates in each environment.

Table 4. Geometric formulae for surface area.

Artificial material	Formula	Shape	Notes
Sheets or strips	$A = L \times W$		L = represents length, W = width

Rectangular prism	$A = 2ab + 2bc + 2ac$		a, b, c = represent the three dimensions of the prism (i.e., length, width and depth)
Ball or spheroid	$A = 4\pi r^2$		r = radius of sphere
Ovoid	$A \approx 4\pi\left(\frac{a^p b^p + a^p c^p + b^p c^p}{3}\right)^{\frac{1}{p}}$		a, b, c = are the three axes of the ellipsoid $p \approx 1.6075$
Filament	$A = 2\pi r L + 2\pi r^2$		L = represents the filament length; for relatively long filaments, the end area (πr^2) may be insignificant
Brushes	$A_\Sigma = N \times A_F$		A_F = represents the filament surface area N = represents number of filaments
Mesh	$A_\Sigma = A_x N_x + A_y N_y - N_x N_y 16r^2$		A_x : Area of the x filaments A_y : Area of the y filaments N_x : Number of x filaments N_y : Number of y filaments r: filament radius (Weisstein 2013)

Part V: Sampling design

Sampling strategies to assess abundance and distribution of benthic organisms have long been a major concern, since changes in sampling designs can lead to very different conclusions regarding species distribution (Statzner et al. 1998, Peterson et al. 2001, Cabral and Murta 2004, Elphick 2008). Even in the context of other benthic biota, the characterization of BHAB dinoflagellate distribution poses a challenging sampling design problem. In addition to the environmental variables that govern abundance and distribution of all HAB species (temperature, salinity, nutrients, etc.), surface association factors (e.g., colonization rates), substrate preferences, migration among different substrates and the complex interactions within the biofilm community may augment the spatial and temporal variability of BHAB dinoflagellates. Added to these effects are factors regulating the macrophyte substrates themselves, which are often characterized by highly patchy distributions (Costa et al. 2001,

Collado-Vides et al. 2007). The net result of all of these considerations is a great deal of spatial-temporal complexity in BHAB cell distribution, making characterization of average BHAB cell abundances problematic.

Collection of field samples of BHAB dinoflagellates has been ongoing in a range of different environments since the early work with *Gambierdiscus* by Yasumoto et al. (1977a,b). This early sampling in the Gambier Islands involved collection and size fractionation of turf algae and detritus, which were scraped from dead corals. While these sampling methods were crude, they were sufficient for the objectives of the study—identification of the source of toxicity in benthic detrital samples (Yasumoto et al. 1977a). During a follow-up study, the authors developed a quantitative sampling method for *Gambierdiscus* cells associated with macroalgae, where dinoflagellate abundance was normalized to macrophyte mass (Yasumoto et al. 1979a). With little improvement, this method provides the basis for nearly all BHAB dinoflagellate sampling conducted to date. It was recognized that collection of multiple macroalgae samples from a location provided a better estimate of average BHAB abundance than if a single sample was collected (Yasumoto et al. 1979a). But the methods for achieving such a "good estimate" have varied from study to study and from location to location because of different BHAB species, different substrates and other site-specific factors. While high spatial variation in BHAB cell abundances has repeatedly been noted (Yasumoto et al. 1979a, Ballantine et al. 1985, Taylor 1985, Taylor and Gustavson 1985, Lobel et al. 1988), there has been little progress in identifying the factors governing differences in small scale BHAB distribution. Why are cell abundances often so patchy? Is this simply an effect of randomness or are other factors involved? If so, at what spatial scales do these factors operate? These are just a few of the questions that have plagued BHAB distributional research in recent decades. Given the progress in experimental design that has characterized marine field sampling since the 1970s, it seems evident that BHAB dinoflagellate field methods need to be brought in line with mainstream ecological sampling. This refinement should include considerations for randomization, replicated sampling and more appropriate experimental designs.

Randomization

A central tenet of quantitative ecological sampling is that individual samples should be collected such that they are independent and randomly distributed. That is, each member of a sample population should have an equal and independent chance of being sampled (Zar 1996, Watt 1998). Such randomization is necessary to minimize the chances that bias during sample collection will influence the data interpretation. To illustrate the potential effects of sampling bias on average BHAB dinoflagellate abundances, consider a small embayment populated by a single species of macroalgae having an approximately homogeneous distribution (Fig. 9). If five replicate macroalgae samples are used to assess mean dinoflagellate abundance using the macrophyte method, which macroalgae should be collected? If the number of BHAB dinoflagellate cells is the same for all macroalgae in the bay, then any five plants will provide the same measure of average abundance. Algae that are collected easily from the shallow water near the dock will be just as useful for assessment of BHAB abundances as those from deeper

water near the bay mouth. Unfortunately, this scenario is seldom the case in natural systems, where an array of environmental factors governs BHAB cell abundances on each plant. If, for instance, the bay is characterized by a north-south environmental gradient (depth, nutrients, salinity, etc.), then macroalgae on the south side might have substantially higher dinoflagellate abundances than those to the north. In such a case, specimens collected in the shallows around the dock might yield a poor estimate of dinoflagellate abundance for the entire bay. This scenario is an example of *selection bias*, which can be defined as a type of systematic error due to non-random sampling of a population. With no *a priori* knowledge of the environmental gradient across the bay, random selection is the only way to overcome the effect of the spatial gradient on the resulting average cell abundance. This is often the case in field studies, where sampling sites are visited briefly, and little information is available about the local environment.

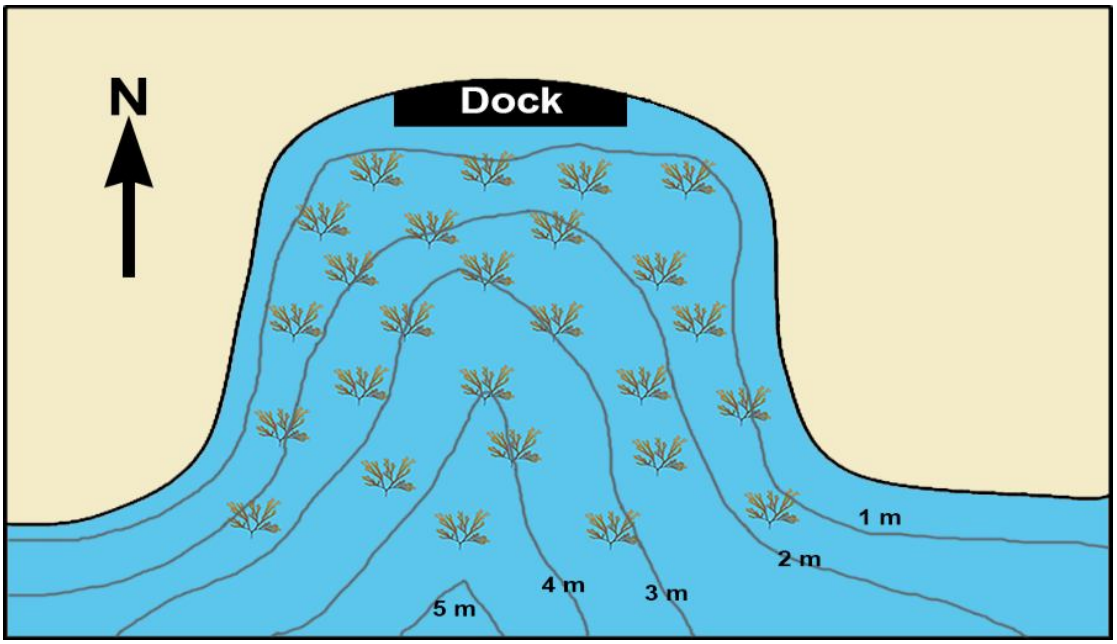


Figure 9. Diagram of small bay with continuous macrophytes distribution. Credit: NOAA.

While the sampling only from the dock in the above scenario may be unlikely in modern field research, more subtle types of selection bias are relatively common and may even be unavoidable. For the macrophyte method, a review of the literature will reveal that samples used to assess BHAB abundance have almost always been selected without randomization. That is, macrophyte specimens are generally selected haphazardly, usually by snorkeling or SCUBA. This method of selection is very susceptible to bias because it relies entirely on the judgment of the collector. Unintended selection bias has been recognized as a very common problem in social or medical studies (Exadaktylos et al. 2013), as well as many types of ecological research (Clarke 1996, Phillips et al. 2002, Martinez and Wool 2006, Zvloni et al. 2008, Phillips et al. 2009).

Some very real scenarios for selection bias involving this type of sampling include: avoidance of macrophytes that are damaged or unhealthy looking; avoiding plants which are too deep, are too far away, or which occur in hard to reach places; excluding those that are dangerous to collect (next to a moray eel den, for instance); and sampling only individuals of a certain size, such as those which fit easily into the sample container. The same may apply if only portions of algal plants are sampled, such as collecting only those branches which occur at the top of the plant or are on the side facing the collector. Some degree of selection bias during BHAB fieldwork is understandable, especially when sampling occurs in remote tropical locations requiring long travel times, in environments with hazardous currents or wave energy, or at locations inhabited by poisonous, venomous, or otherwise dangerous marine life. Only by random selection of macrophyte samples can the effects of such biases be minimized. Randomized sampling is designed to minimize sampling bias because it eliminates the judgment of the individual during the collection process. Although the need for randomization is widely recognized, in practice the process of selecting random samples is often far from trivial and may be very challenging in remote field settings. The sections below detail some of the most common random sampling methods and their adaptation to BHAB research.

Replication and sample size

Another basic principle of ecological sampling, indeed of all sampling, is replication. The functions of sample replication are to reduce the effect of random variation on the average (or other measure of central tendency) and to provide some assessment of the reliability of the measurements involved by quantifying the differences among replicates (Lenth 2001, Cumming 2006). But just how many macrophyte samples need to be collected in order to determine average BHAB dinoflagellate abundance? While the scope of this chapter is insufficient to cover much of the theoretical basis behind determining the sample size necessary for BHAB field studies, it would be useful to summarize some of the more practical considerations with an example from fieldwork. For more detailed information about the statistical theory and techniques used to estimate sufficient sample sizes, the reader is referred elsewhere (Lobel et al. 1988, Sokal and Rohlf 1995, Zar 1996, Pillar 1998, Lenth 2001, Lewis 2006, Williams et al. 2007, Bacchetti et al. 2008, Coe 2008, Bacchetti 2010, Barbiero et al. 2011).

Consider an example from field sampling—the abundance of *Prorocentrum* cells associated with the phaeophyte *Padina* sp. collected at a site in Malaysia during May of 2012 (GEOHAB Workshop 2012, Tester et al. 2014). At this site, six specimens were randomly sampled within a small *Padina* bed and *Prorocentrum* cell abundances were determined per gram of algae using the macrophyte method and microscopy (Table 5). A typical research question might be: Does the abundance of *Prorocentrum* cells at this site differ significantly from another location? In the context of this section, the question is better phrased: Is a sample size of six macroalgae sufficient to resolve the mean abundance of *Prorocentrum* from the abundances at other sites? The field data showed *Prorocentrum* abundances from the six macroalgal specimens ranged between 83 and 217 cells g^{-1} (wet weight) of *Padina* with a mean and

variance of 145 ± 2,524 cells g^{-1} (Table 5). A common method for estimating sample size (n) derived from a Student's t-test requires the sample variance (s^2), a desired level of statistical power (β) and some specified difference among two sample means (δ) as in Equation 1 (Zar 1996). The term

$$n = \frac{s^2}{\delta^2}(t_{\alpha,(2),v} + t_{\beta(1),v})^2 \qquad \text{Eq. 1}$$

$t_{\alpha,(2),v}$ denotes the 2-tailed table t-value at the α = 0.05 significance level, where v is degrees of freedom (n-1). The term $t_{\beta,(1),v}$ denotes the 1-tailed table t-value at the α = 1-β level, where β is the statistical power. With a common level of statistical power of 0.90 (i.e., the probability [90%] of correctly rejecting a false hypothesis, Zar 1996), a reasonable difference among the two sample means of 100 cells g^{-1} and the sample variance (2,525.2 cells 100 cm^{-2}), $t_{\alpha,(2),v}$ = 2.571 and $t_{\beta,(1),v}$ = 1.476 (t-table from Zar 1996), the calculated sample size is 4.1. This result indicates four replicate samples of *Padina* would be sufficient to resolve differences in *Prorocentrum* abundance among two sites where mean abundances differ by 100 cells g^{-1}.

Table 5. *Prorocentrum* abundances on replicate macrophyte samples collected 23 May, 2012 at Pulau Sibu, Malaysia. Data from Tester et al. (2014).

	Sample No.	**Cells g^{-1}**	
Site 1 *Prorocentrum* spp.	1	125.8	Mean 145.0 Variance 2525.2 Stdev 50.2 CV 34.6 %
	2	82.9	
	3	113.0	
	4	217.5	
	5	190.8	
	6	140.2	

But how informative is this estimate? And what value of δ is reasonable? If power is fixed at 0.90, then the number of samples required depends on the sample variance and the difference among the two means. If a smaller difference of only 50 cells g^{-1} is allowed, the required sample size increases to 17 *Padina* specimens, a more daunting prospect for sampling, especially in a relatively small patch of algae. For this same example, other sites in the vicinity exhibited mean *Prorocentrum* abundances of 73-269 cells g^{-1}, so the initial δ value of ±100 cells g^{-1} seems a reasonable allowance to estimate sample size. It should be recognized that the "correct" sample size depends upon the particular hypothesis that is being tested, the degree of statistical rigor that is allowed (i.e., α = 0.01 or 0.1 instead of 0.05), the natural variability among samples and ultimately, upon the objectives of the study. There are no defining thresholds for sample variability, statistical significance or power; these quantities are highly relative (Bacchetti 2010). Perhaps the best that can be learned from this particular example is the importance of preliminary information about a site to gauge the expected range of abundances and degree of variability among samples before an appropriate sample size (and sampling design) can be formulated.

There are other practical considerations that must be weighed during sample size determination for a BHAB field study. Where the macrophyte method is used, a limiting factor may be the number and distribution of macroalgae available for sampling. What if the estimated sample size is ten macroalgae, for instance, but only five specimens are present? One solution is to collect all five macroalgal specimens and accept that the calculated mean abundance may be inadequate to characterize the site. Another solution is to use a substrate other than macroalgae, such as coral debris, seagrass or sediment to assess abundance. Another alternative is to employ an artificial substrate, replicates of which can be deployed across the site in any number. Some more practical considerations in determining sample size are the amount of time and number of support personnel available for sample collection, processing and cell enumeration, especially if multiple sites are sampled. In the above example from Malaysia, a sample size of six was established in advance due to personnel and time constraints. For more information about sample size considerations the reader is directed to other sources (Pillar 1998, Lenth 2001, Williams et al. 2007, Bacchetti et al. 2008, Coe 2008, Bacchetti 2010).

Types of sampling

Sampling designs for BHAB dinoflagellates have been focused primarily on spatial or temporal characterization of dinoflagellate distribution, abundance and/or diversity. Here, the focus will be on spatial characterization of BHAB populations, although many of the details are applicable to temporal studies as well. As stated above, designs utilized for BHAB sampling have been fairly simple, and new approaches are needed to both incorporate randomization and replication. Because BHAB dinoflagellates are predominantly associated with substrate surfaces and sediment, mainstream benthic ecology can provide sampling designs that are directly or indirectly adaptable to collection and/or enumeration of BHAB species.

Perhaps the simplest form of sampling can be termed *accidental sampling*, which is collection of samples of opportunity with little planning or regard for sampling design. A few pieces of drift algae collected along a hotel beach, a plastic bag of coral debris collected at a vacation spot or a seagrass sample collected during a kayaking trip are all examples of this type of sampling. Such samples are often collected in an assortment of containers, which include plastic sandwich bags, food jars and shampoo or soft drink bottles. Because sampling is largely unplanned, there is typically no randomization or replication, and accidental samples are often qualitative in nature. Nonetheless, this simple type of sample collection has made substantial contributions to BHAB ecology and has led to the discovery of new dinoflagellate species. Some examples include the discovery of *Coolia tropicalis* from some discolored sand in Belize, Central America (Faust 1995) and isolation of *Gambierdiscus carolinianus* from a few macroalgae samples collected during reef fish surveys along the coast of North Carolina, USA (Litaker et al. 2009). Although useful information may be gleaned by accidental sampling, there is little environmental context accompanying the samples and no hypotheses can be

addressed. Perhaps the greatest benefit from accidental sampling is that results may guide more quantitative sampling in follow-up studies.

Randomized sampling

A more useful type of BHAB sample collection is *randomized sampling*, where substrate samples are identified and collected at random across the spatial landscape of the site of interest. The least structured random design is termed *fully randomized sampling*, where sample sites are completely independent and chosen without any level of spatial organization. The sample locations are typically identified in advance using a map or grid. Some examples include choosing coordinates on a gridded map, selecting latitude and longitude pairs or picking out random pixels from an aerial photo or satellite image. Fully randomized designs may be best applied to environments with large areas of visually uniform characteristics, such as seagrass beds or sand flats when no preliminary surveys of the site of interest have been completed (Webb and Parsons 1992, Lessios 1996, Govindasamy and Anantharaj 2012). The benefits of fully randomized sampling are that the method is very easy to apply, providing suitable maps or images are available. For example, high resolution maps of benthic habitat types provide useful guidance for placement of sampling locations with regard to benthic cover, sediment type, depth, or other parameters (Fig. 10). Other examples of suitable maps of tropical benthic habitats are those provided by the United Nations Environmental Program (UNEP-WCMC 2013, http://data.unep-wcmc.org) or via the NOAA Center for Coastal Monitoring and Assessment (CCMA 2013, http://ccma.nos.noaa.gov/products/biogeography/benthic/default.aspx). More examples of where this technique was applied include studies that used remote sensing imagery (Kendall et al. 2001, Bruckner and Renaud 2012) and acoustic mapping data (Clements et al. 2010, Kostylev 2012). Unfortunately, such detailed spatial maps are often available only for designated preserves, sanctuaries, marine parks or other high-profile sites. Resources may be limited in more remote regions and other methods may be necessary.

Another problem with fully randomized designs is that the benthic biota of interest typically have patchy distributions characterized by high spatial variability. As a result, large numbers of samples may be necessary to estimate mean abundance with reasonable confidence intervals. For example, Chutter (1972) found that 112 individual sediment samples were necessary to estimate the abundance of benthic invertebrates within a small homogenous streambed with a confidence of ±10% of the mean and that 448 were required for ±5%. Clearly, selecting fully random samples when such variability occurs might be impractical or else larger margins of error might be more appropriate (Pillar 1998). For example, the recent study by Tester et al. (2014) considered a coefficient of variation of 0-100% as a reference point to compare variability in BHAB dinoflagellate abundances among natural and artificial substrates.

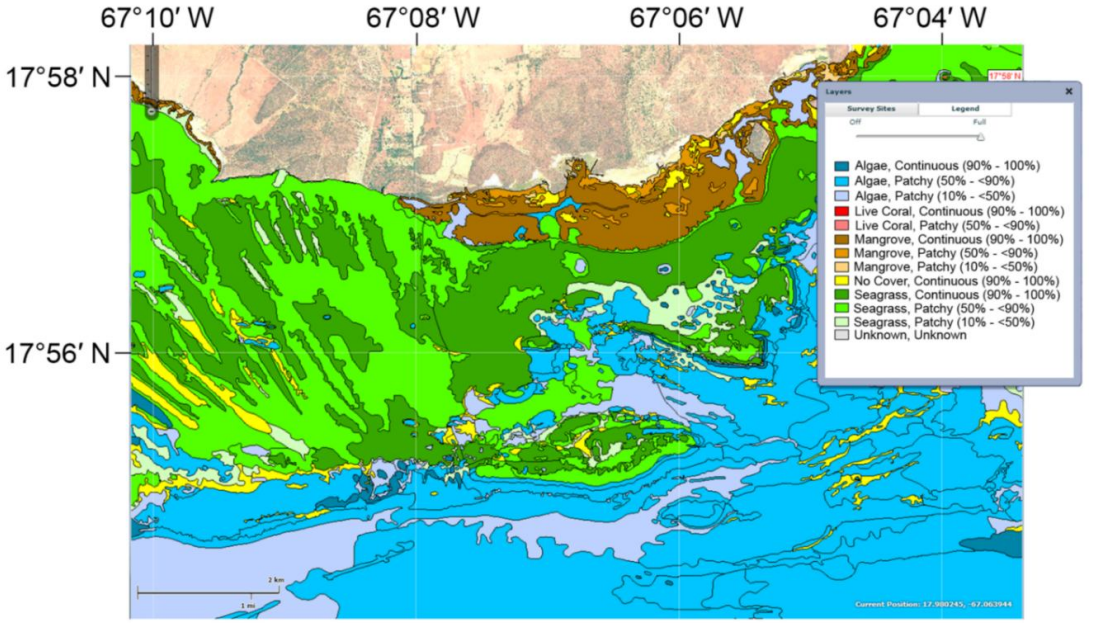


Figure 10. Map of vegetation types along the southwest coast of Puerto Rico, USA. Image from the Southwestern Puerto Rico BIOMapper website: http://maps.coastalscience.noaa.gov/biomapper/biomapper.html?id=SWPR.

An alternative to fully randomized design is *categorized sampling* (stratified sampling), where potential sampling sites are divided into sub-environments with unifying characteristics. Such categories might include sites of the same depth, bottom type or wave energy; sites having the same benthic biota, such as corals, seagrass or mangroves; sites within the same geographic location or having the same sediment type. Categorized approaches were developed to reduce variability among samples by eliminating confounding environmental factors among different types of sites. Typically, sample locations are pre-identified within appropriate categories and then a number of sample locations in each category are randomly selected. For example, Richlen and Lobel (2011) categorized sampling sites according to habitat type, water depth and flow characteristics prior to sampling for BHAB dinoflagellates. This method has also been successfully applied to the distribution of other biota, such as macroalgae on coral reefs (Phillips et al. 1997), estuarine fouling biota (Ardisson and Bourget 1992), and intertidal microgastropods (Barnes 2010).

A similar approach, *cluster sampling*, is used to account for the patchiness of certain substrates. For example, some macrophytes, such as seagrasses or macroalgae tend to occur in beds or patches, as do invertebrates like corals, sponges and shellfish. Relevant clusters of biota are pre-identified and then individual samples are randomly selected within each cluster to reduce variability among clusters. Occurrence of BHAB dinoflagellates in such patches is common due to apparent preference for certain macroalgal hosts (themselves occurring in clumps), spatial heterogeneity of hydrographic characteristics that govern passive settling of cells, *in situ* growth and mortality of the cells on the substrates, and migration of BHAB cells

among substrates. Cluster sampling was utilized recently by Tester et al. (2014) for BHAB dinoflagellate sampling in Malaysia.

A more powerful type of categorized designs is *hierarchical sampling*, where categories, are themselves nested within larger groups. The statistical differences within and among categories may then be analyzed much like a nested analysis of variance. The benefit of hierarchical sampling is that several levels of comparisons may be made at the same time and that the most important spatial scales of variation may then be identified. For instance, a study may examine differences in BHAB abundance among three seagrass beds on the leeward side of an island. The leeward seagrasses may be compared to a group of beds on the windward side of the same island as well as grass beds on different islands. In this case, there is variability among islands, among leeward and windward locations, among each grass bed and among replicate samples within each bed. The nesting of levels within the hierarchy addresses variability at a range of spatial scales from a few meters (replicates in each grass bed) to multiple kilometers (among islands). Many such sampling designs may be devised providing the hierarchy conforms to some spatial, temporal or ecological scheme. Although hierarchical sampling has not yet been applied in BHAB research, the technique is common in other types of ecology (e.g., Edgar and Klumpp 2003, Pardi et al. 2006, Sabater and Tofaeono 2007). The importance of spatial scales and the related ecological context of hierarchies have been reviewed in Levin (1992), Dethier and Schooch (2005), and Fraschetti et al. (2005).

Linear sampling designs

Linear designed sampling schemes are one of the most common methods employed to sample aquatic systems. In general, sample locations are selected along a line of specified length (transect) stretching across the site of interest. Transects may take the form of an actual line or may be widened into a swath or belt (Buckland et al. 1993). This approach is often applied when a known gradient, such as water depth, salinity or bottom type occurs across a study site. Sample sites may be at specified locations along the transect line or may be arranged within boundaries on either side of the lines. Samples may be collected systematically or at random, much like placement of the transects themselves. Some common approaches include arrangement of transects perpendicular to a shoreline (Adjeroud 1997, Giménez et al. 2010), stretching across features such as coral reefs or grass beds (Hill and Wilkinson 2004, Jokiel et al. 2005), or at specified depths or other locations parallel to the shoreline (Figs. 11A-C) (Kennison et al. 2003, Crabbe 2008). Samples collected along each transect can then be analyzed with linear regression, nested ANOVA or in a variety of other ways. Randomization of samples is generally achieved during transect placement (starting point, direction, etc.) and/or during designation of sample locations along the transect. Two of the most common types of applications are line-intercept samples, those which directly intersect the transect path (Hewitt et al. 2002, Nunes et al. 2011); and quadrat samples, where quadrats either intersect the transect line or are placed at locations along the sides (Shears and Ross 2009, Wallenstein et al. 2009, Seoane et al. 2012) (Figs. 11D-F). Transects have also been utilized for suction sampling of biota (Samu et al. 1997, Prussian et al. 1999, Ramsdale et al. 2011). The use of

linear transect designs has become predominant in coral reef research, where the spatial coverage of macrophytes, corals and benthic invertebrates are routinely quantified along linear transects spanning reef systems (Kendall et al. 2003, Miller et al. 2003, Crabbe 2008). Although many studies are still completed using visual transects by divers (Kennison et al. 2003, Brown et al. 2004, Ayotte et al. 2011), increasingly sonar, photo, video and remote sensing methods are used to supplement or replace the manual methods (Kendall et al. 2003, Roelfsema and Phinn 2009, Bruckner and Renaud 2012, Kostylev 2012, Seoane et al. 2012). These remote methods enable distributional mapping of larger geographic areas than would be possible by traditional survey methods. Remote optical methods typically require spatial validation using randomized, categorized, and/or hierarchical sampling methods (Coles et al. 1993, Walker et al. 2008, Clements et al. 2010, Seoane et al. 2012) and are of limited utility in water deeper than ~20 m. Such benthic survey methods can be useful for identifying spatial distribution of BHAB substrates (seagrasses, macroalgae, etc.) and can be easily adapted for dinoflagellate sample collection.

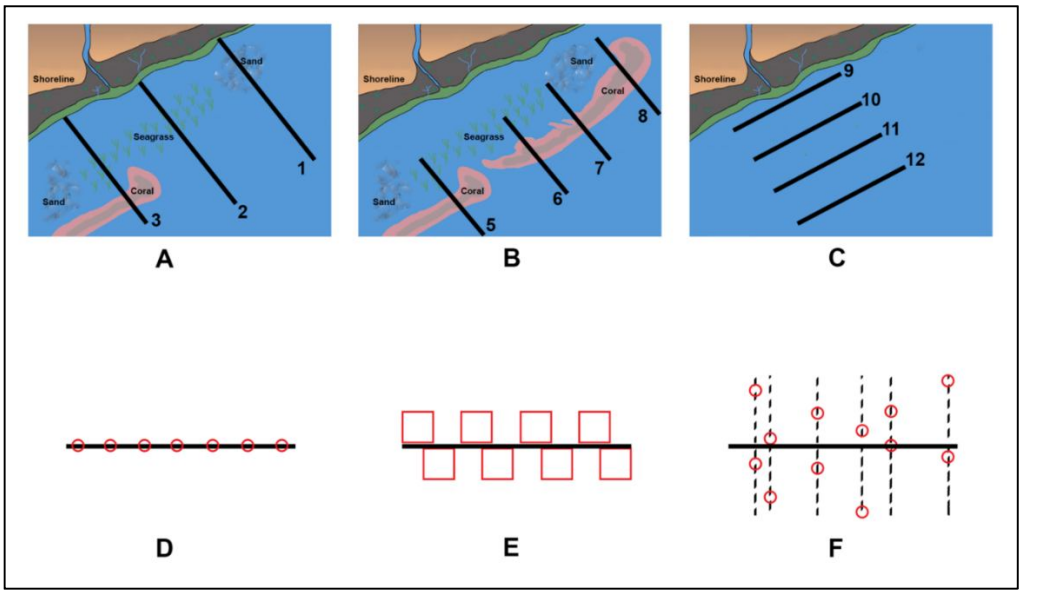


Figure 11. A variety of different transect sampling designs adaptable for BHAB sampling. **A.** Transect lines perpendicular to the shore. **B.** Transect lines across benthic features. **C.** Lines parallel to the shoreline, alternatively along depth contours. **D.** Line intercept transect design where sampling sites are located on the transect. **E.** Quadrat transect design where defined areal plots are located at fixed points along the transect line. **F.** Randomized transect design where sample locations are located at random distances along perpendiculars to the mail transect. In this case, the perpendiculars are also located at random distances along the transect. Credit: NOAA.

It is important to note that there is a potential statistical problem with transect approaches if care is not taken during the analysis. Namely, samples distributed along a given transect are not completely independent because of their spatial association. Treating points on the same transect as individual samples has been identified as a type of pseudoreplication (Lessios 1996), which occurs when either treatments are not replicated (though samples may be) or

replicates are not statistically independent (Hurlbert 1984). Pseudoreplication may be avoided by considering the entire transect as an experimental unit and then using multiple, randomly placed transects. This approach can be advantageous for studies seeking to assess the overall abundance at a site because transects can extend across a range of microenvironments, thus providing a better average when multiple transects are used. Transect pseudoreplication may also be alleviated with more complex statistical methods, such as mixed-effect models or random slope designs (Millar and Anderson 2004, Schielzeth and Forstmeier 2009). It should be noted that the pseudoreplication problem is also applicable to many systematic surveys, where measurements are taken at pre-selected, spatially dependent sites. Because each sample point is located with respect to adjacent points, the individual samples are not entirely independent. Such spatial linkage can be relieved by the use of certain types of Kriging (Petitgas 1993, Bez 2004, Millar and Anderson 2004). Ultimately, analysis with spatial statistical methods may be required to characterize the degree of linkage (spatial autocorrelation) among adjacent data points and to assess the underlying hypotheses about distribution (Legendre 1993, Lloyd et al. 2005, Bataineh et al. 2006).

Part VI: Conclusions

With the recent resurgence of interest in BHABs, the lack of standardized sampling protocols is a major impediment to statistically valid studies and reduces the potential for meaningful comparison between studies. This is a prime concern voiced across the entire community (GEOHAB Workshop 2012). The use of artificial substrate is posited as one solution and allows BHAB cells to be normalized across habitats and among studies. This sampling method is based on the observation that benthic HAB species migrate into the water column and colonize new substrates over short distances. This leads to the testable hypothesis that benthic cells recruit to an artificial substrate in proportion to the overall density of the cells in the surrounding habitat. An artificial substrate as common as window screen has the advantage of being relatively easy to deploy and allows samples to be collected from any location, including the water column. Using artificial substrates, hypothesis testing can be accomplished in diverse habitats supported by statistically robust sampling designs that include randomization and true replicate samples. Fortunately, identification and semi-quantification of many of the BHAB species has been made more tractable with the use of molecular methods. While qPCR assays are tedious and demanding to develop, once validated, they are powerful tools. Coupled with improved sampling methods, researchers are now strategically positioned to ask sophisticated questions about factors that affect the physiology, toxicity and toxin transfer of BHAB species.

Acknowledgements

We would like to dedicate this chapter to Dr. Maria Faust for sharing her knowledge of benthic dinoflagellates with us and inspiring their collection with artificial substrates. The Scientific Committee on Oceanic Research (SCOR) and Intergovernmental Oceanographic Commission

of UNESCO's Global Harmful Algal Blooms (GEOHAB), HABs in Benthic Systems Open Science Meeting in Honolulu, HI, in June 2010 provided an opportunity to bring problems related to standardized sampling methods for benthic HAB species into focus across the BHAB community. The Smithsonian Institution's Caribbean Coral Reef Ecosystem Program, NOAA's International Programs Office and the GEOHAB Core Research Project funded by Intergovernmental Oceanographic Commission of UNESCO -Yeosu Project of Korea all provided field opportunities for testing artificial substrates to collect BHAB species. Partial funding was provided as an award from the National Oceanic and Atmospheric Administration's Ecology and Oceanography of Harmful Algal Blooms program for the development of the artificial substrate method.

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