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1 Development of Fluorescence in situ Hybridization (FISH) Probes to Detect and Enumerate
2 *Gambierdiscus* Species
3

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16 **Highlights**

17 ● FISH probes developed to detect Caribbean and Pacific species of *Gambierdiscus* in field
18 samples.
19 ● Multiple *Gambierdiscus* species shown to co-occur in natural populations in the Caribbean
20 and the Pacific.
21 ● FISH probes detected temporal changes in *Gambierdiscus* community composition in the
22 Florida Keys.
23

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24 **Abstract**

25 Ciguatera fish poisoning (CFP) is a syndrome caused by the bioaccumulation of
26 lipophilic ciguatoxins in coral reef fish and their subsequent consumption by humans. These
27 phycotoxins are produced by *Gambierdiscus* spp., tropical epiphytic dinoflagellates that live on a
28 variety of macrophytes, as well as on dead corals and sand. Recent taxonomic studies have
29 identified novel diversity within the *Gambierdiscus* genus, with at least 18 species and several
30 sub-groups now identified, many of which co-occur and differ significantly in toxicity. The
31 ability to accurately and quickly distinguish *Gambierdiscus* species in field samples and
32 determine community composition and abundance is central to assessing CFP risk, yet most
33 *Gambierdiscus* species are indistinguishable using light microscopy and other enumeration
34 methods are semi-quantitative. In order to investigate the spatial and temporal dynamics of
35 *Gambierdiscus* species and community toxicity, new tools for species identification and
36 enumeration in field samples are needed. Here, fluorescence *in situ* hybridization (FISH) probes
37 were designed for seven species commonly found in the Caribbean Sea and Pacific Ocean,
38 permitting their enumeration in field samples using epifluorescence microscopy. This technique
39 enables the assessment of community composition and accurate determination of cell
40 abundances of individual species. Molecular probes detecting *G. australes*, *G. belizeanus, G.*
41 *caribaeus*, *G. carolinianus, G. carpenteri,* and the *G. silvae/G. polynesiensis* clade were
42 designed using alignments of large subunit ribosomal RNA (rRNA) sequences. These probes
43 were tested for specificity and cross-reactivity using *Gambierdiscus* cultures, and a series of
44 experiments were performed in which field samples spiked with known concentrations of
45 *Gambierdiscus* cultures were analyzed to confirm that *Gambierdiscus* can be successfully
46 detected and enumerated by FISH in the presence of detritus and other organisms. These probes

47 were then used to characterize *Gambierdiscus* community structure of field samples collected
48 from the Florida Keys and Hawai‘i, USA. The probes revealed the co-occurrence of multiple
49 species at each location. Time-series FISH analyses of samples collected from the Florida Keys
50 quantified seasonal shifts in community composition as well as fluctuations in overall
51 *Gambierdiscus* cell abundance. Application of species-specific FISH probes provides a powerful
52 new tool to those seeking to target individual *Gambierdiscus* species, including significant toxin-
53 producers, in field populations. Moving forward, analysis of *Gambierdiscus* community
54 composition across multiple environments and over time will also allow species dynamics to be
55 linked to environmental parameters, improving our ability to understand and manage the current
56 and changing risks of CFP worldwide.
57
58
59 **Abbreviations**
60 CFP, ciguatera fish poisoning; FISH, fluorescence *in situ* hybridization; qPCR, quantitative
61 polymerase chain reaction; LSU rDNA, large subunit ribosomal DNA; LSU rRNA, large subunit
62 ribosomal RNA; USVI, US Virgin Islands
63
64 **Keywords**
65 Ciguatera, *Gambierdiscus*, ciguatoxin, fluorescence in situ hybridization, harmful algal bloom
66
67 **1. Introduction**
68 Ciguatera fish poisoning (CFP) is a syndrome caused by the bioaccumulation of
69 lipophilic ciguatoxins in coral reef fish and subsequent consumption by humans (Scheuer et al.,

70 1967; Lehane and Lewis, 2000). These phycotoxins are produced by members of the genus
71 *Gambierdiscus*, a tropical epiphytic dinoflagellate genus that lives on many varieties of
72 macroalgae but also may occur on dead corals and sand (reviewed in Parsons et al., 2012).
73 Different species of *Gambierdiscus* have been shown to exhibit algal host preferences, and high
74 densities of cells have been found on diverse algal taxa including calcified species (*Jania*),
75 brown algae (*Dictyota*), green algae (*Chaetomorpha*) and turf algae (Rains and Parsons, 2015;
76 Cruz-Rivera and Villareal, 2006). Species of *Gambierdiscus* have also been shown to have
77 different growth characteristics and toxin-producing capabilities, topics that continue to be
78 actively researched (Xu et al., 2016; Kibler et al., 2012; Litaker et al., 2017). Given the
79 substantial physiological and toxicological diversity within the genus, risk of CFP may be largely
80 determined by *Gambierdiscus* species community composition, their distribution and abundance
81 across available benthic habitat, and the prevalence of certain toxic species or strains.

82 As *Gambierdiscus* species are almost indistinguishable under the light microscope,
83 current techniques to identify cells to the species level include scanning electron microscopy,
84 sequencing of large subunit ribosomal DNA (LSU rDNA) sequences, and recently developed
85 restriction fragment length polymorphism (RFLP) typing methods (Lyu et al., 2017; Lozano-
86 Duque et al., 2018). Semi-quantitative qPCR has also been developed for large scale analysis of
87 field samples (Vandersea et al., 2012), but it does not have the same quantitative strength as
88 microscope counts due to varying levels of rDNA within *Gambierdiscus* cells as well as possible
89 matrix effects (currently under evaluation; D. Anderson, unpublished data). Species-specific
90 fluorescence *in situ* hybridization (FISH) probes offer an alternative approach, allowing for the
91 determination of *Gambierdiscus* community composition and abundance in field samples while
92 preserving the accuracy of microscopic cell counts. This differentiation of *Gambierdiscus*

93 species allows the direct elaboration of community dynamics and shifting patterns of community
94 toxicity, and thus provides a valuable tool for monitoring toxic species.

95 Molecular FISH probes targeting LSU rRNA have long been used in monitoring bloom
96 populations of *Alexandrium* species associated with paralytic shellfish poisoning (PSP) in the
97 Gulf of Maine region, as well as in the detection and enumeration of other harmful algal bloom
98 species (e.g., Anderson et al., 2005; Chen et al. 2008; Mikulski et al., 2005; Parsons et al., 1999;
99 Scholin et al., 1996). In cases where the target taxon is only a minor member of a phytoplankton
100 assemblage or where there may be detritus in field samples, as is true in both cases for
101 *Gambierdiscus*, FISH probes can enable accurate identification of cells (Anderson et al., 2005).
102 In this study, probes detecting *G. australes*, *G. belizeanus, G. caribaeus*, *G. carolinianus, G.*
103 *carpenteri*, and the *G. polynesiensis* clade (*G. polynesiensis* and *G. silvae*) were designed using
104 alignments of large subunit ribosomal RNA (rRNA) sequences in the D1-D3 and D8-D10
105 regions of the LSU rRNA gene. These species were selected based on their common occurrence
106 in the Caribbean Sea and/or Pacific Ocean, and in the case of *G. polynesiensis* and *G. silvae,*
107 their high toxicity (Chinain et al. 1999, 2010; Longo et al., 2019; Robertson et al,. 2018).
108 Candidate probes were tested for specificity and cross-reactivity using cultured isolates that
109 included both target and non-target species, and a series of experiments were performed in which
110 field samples from St. Thomas, USVI were spiked with known concentrations of *Gambierdiscus*
111 cultures were analyzed to confirm that *Gambierdiscus* can be successfully detected and
112 enumerated by FISH in the presence of detritus and other organisms. These probes were then
113 used to characterize *Gambierdiscus* community structure of field samples collected from the
114 Florida Keys and Hawai‘i, USA.

115 The genus *Gambierdiscus* has a circumtropical distribution, but individual species can
116 have more limited ranges (Lewis 2001; Litaker et al., 2010). Florida lies on the northern edge of
117 ciguatera incidence although *Gambierdiscus* species have been isolated as far north as off the
118 coast of North Carolina (Litaker et al,. 2009; Radke et al., 2015). Hawai‘i also has a relatively
119 low incidence of ciguatera compared to other Pacific islands even though multiple species of
120 *Gambierdiscus* have been reported (Skinner et al., 2011; Litaker et al., 2010). Conversely, St,
121 Thomas, USVI, historically has had high incidences of ciguatera poisoning and much work has
122 gone into forecasting ciguatera risk for the region (e.g., Radke et al. 2013). In this study we take
123 advantage of field samples and cultured isolates collected from all three locations to test
124 *Gambierdiscus* probes against various species assemblages and substrate types. Molecular
125 probes were applied both individually and in combination using different fluorophores,
126 permitting multiplexing in subsequent analyses of field samples.

127 Given the potential for variable toxicity among co-occurring *Gambierdiscus*, species, it is
128 now commonly accepted that species composition can have a greater influence on risk of CFP
129 than total genus abundance, as high local abundance of *Gambierdiscus* cells has not always led
130 to a CFP events (discussed in Litaker et al., 2010). The hypothesized link between ciguatera
131 outbreaks and the toxicity of individual species was first proposed by Holmes et al. (1991) and
132 Legrand (1998), suggesting that certain “super-producing” strains of *Gambierdiscus* were
133 responsible for high ciguatoxin levels leading to outbreaks. The discovery of highly toxic
134 species such as *G. polynesiensis*, *G. silvae*, and *G. excentricus* lend support to these early
135 hypotheses. The approach outlined herein allows for accurate, quantitative determination of
136 community composition, as well as the selective enumeration of species known to exhibit high
137 toxicity. Species abundance measurements can be integrated into routine monitoring efforts and

138 used to investigate community structure over spatiotemporal gradients, allowing establishment of
139 seasonal patterns, or elucidation of community response to disturbance events such as storms or
140 coral bleaching.
141
142 **2. Materials and Methods**
143 2.1 *Probe Design and Testing Overview*
144 Fluorescent probes were designed to target the large subunit (LSU) region of rRNA of
145 seven species of *Gambierdiscus: G. australes*, *G. belizeanus, G. caribaeus*, *G. carolinianus, G.*
146 *carpenteri, G. polynesiensis* and *G. silvae*. DNA sequences from the D1-D3 and D8-D10
147 domains of the LSU were obtained from GenBank (www.ncbi.nlm.nih.gov/genbank/) and
148 aligned. Regions of conservation within species were identified, and candidate probes were
149 designed that contained at least two nucleotides of difference between species (Table 1). Every
150 effort was made to design probes with similar melt temperatures (Tms) to aid in multiplexing.
151 Candidate probes were conjugated to a fluorophore for initial testing (Cy3 or FITC) using
152 cultures of *Gambierdiscus*. Based on their availability and viability in culture, eight
153 *Gambierdiscus* species and one ribotype were included in this testing (Table 2): *G. australes*, *G.*
154 *belizeanus, G. caribaeus*, *G. carolinianus*, *G. carpenteri*, *G. pacificus*, *G. polynesiensis*, *G.*
155 *silvae*, and *Gambierdiscus* sp. ribotype 2.
156 During initial testing, cultures were hybridized in separate reactions within a
157 hybridization manifold with individual candidate probes labeled with either a Cy3 or FITC
158 fluorophore, which were cost-effective for candidate probe screening. These experiments
159 included a separate reaction with a universal probe targeting small subunit (SSU) rRNA (Univ-
160 1390, Zheng et al., 1996), which served as a positive control. Each test also included a negative

161 control reaction (no probe). These positive and negative control reactions were used to confirm
162 that the culture cells' rRNA complement was compatible with FISH probe labeling, and that
163 probe signal could successfully distinguish a labeled cell from unlabeled cells. Specific
164 preservation and hybridization steps are detailed in Sections 2.3-2.5, and involved concentrating
165 formalin/methanol preserved samples on cyclopore filters, followed by examination using
166 epifluorescence microscopy. Subsequent to candidate probe testing, probe labeling was also
167 carried out using Alexa Fluor® dyes (see Section 2.2), which provided a brighter signal in field
168 samples. All samples in this study were analyzed using a Zeiss Axio Vert.A1 inverted
169 microscope (Carl Zeiss AG, Oberkochen, Germany) at 100×.
170 Subsequent to initial cross-reactivity testing described in Section 2.4, experiments were
171 performed in which preserved aliquots of cell cultures with a known concentration were added to
172 field samples known to be devoid of *Gambierdiscus* cells (see Section 2.5). These samples were
173 hybridized and enumerated, and compared to cell culture concentrations to verify that the method
174 provides a quantitative assessment of *Gambierdiscus* species abundance.
175
176 2.2. *Overview of multiplexing approach for detecting multiple species in field samples*
177 To enable enumeration of multiple species within a sample, probes that were successfully
178 tested for cross-reactivity were combined into groups based on the region of their target species
179 (Caribbean or Pacific), as well as their Tms, which ranged from 52 to 55°C (Table 1). In order to
180 best combine probes into regional groups for field sample analysis, probes with most similar
181 Tms were combined. Probes that were grouped in two different combinations at different Tms
182 (*G. carpenteri* and *G. caribaeus*, both present in Caribbean and Pacific) were tested at both Tms
183 to ensure they still maintained specificity at a slightly different Tm. Available laboratory

184 microscope filters (FITC long pass, Zeiss 09, Cy3 long pass, Chroma 49016, Tx Red long pass,
185 Chroma 19006) enabled three different fluorophores to be used simultaneously in the same
186 reaction. Given the availability of three flours and the requirement to screen five species within
187 both Pacific and Caribbean regions, each sample was split and screened with either the Group I
188 or Group II species-specific probes (Table 1). Calcafluor White M2R cellulose stain (Sigma-
189 Aldrich, MO, USA), which renders phytoplankton cells visible under the DAPI filter set (Fritz
190 and Triemer, 1985), was applied in the final step of the hybridization process (see Section 2.4) to
191 facilitate initial identification of *Gambierdiscus* cells to the genus level as well as enumeration of
192 any unlabeled *Gambierdiscus* in field samples.

193 When analyzing multiplexed samples, each slide was initially scanned under the DAPI
194 filter set. Whenever a *Gambierdiscus* cell was encountered, the Cy3, TxRd, and FITC filters
195 were applied in sequence in order to determine whether the cell was labeled with a fluorophore.
196 The filter set was then returned to DAPI, and scanning continued until another *Gambierdiscus*
197 cell was located. Depending on density of sample contents and quantity of *Gambierdiscus,* time
198 required for a fully trained personnel to analyze 1 mL in a Sedgwick-rafter slide averaged around
199 20 minutes.

200 Initially Cy3 was used for groups of two probes, but was replaced by Alexa Fluor® 532
201 in the groups of three probes, as the Cy3 fluorescence caused some overlap within the Texas Red
202 excitation filter, and Cy3 labeled cells exhibited higher autofluorescence under the Texas Red
203 filter compared with Alexa Fluor® 532. However, since Cy3 is less expensive and still effective
204 in combination with Alexa Fluor® 488, it was kept for the two-member group. Both options are
205 listed in Table 1. Alexa Fluor® 488 was used in place of FITC, and Alexa Fluor® 594 in place of
206 Texas Red fluorophores that were used in initial culture testing as they produced a brighter signal

207 that was easier to detect over detritus that can have varying levels of autofluorescence under
208 different filters. This is a common issue with FISH labeling of field samples, which was also
209 combated through the addition of Calcafluor White stain during sample processing to facilitate
210 initial identification of *Gambierdiscus* cells to the genus level. This two-group multiplexing
211 approach enabled visualization of multiple *Gambierdiscus* species at the same time, and was
212 used for subsequent analysis of field samples (Section 2.6). Specifics regarding procedures for
213 sample preservation, processing, and analysis are detailed below.
214
215 2.3. *Sample Preservation*
216 Sample preservation procedures were identical for both cultured isolates and field
217 samples, and followed those described in Anderson et al. (2005). Each 14 mL sample was
218 preserved with 750 µl of formalin and stored for at least five minutes, but no more than 24 hours.
219 The sample was then centrifuged at 3000 *g* for 10 minutes, the supernatant removed via
220 aspiration, and each sample was then resuspended in ice-cold methanol (volume was adjusted to
221 accommodate sample density). Samples were stored at -20°C for at least 24 hours prior to whole
222 cell hybridization. Prompt and proper preservation of samples is extremely important, otherwise
223 cells may exhibit high levels of autofluorescence which can interfere with probe visualization.
224
225 2.4. *Cross-reactivity testing using cultures and field samples*
226 The *Gambierdiscus* cultures used in cross-reactivity testing were labeled with FISH
227 probes following a whole-cell hybridization method adapted from Anderson et al. (2005), which
228 involved collecting samples on a cyclopore membrane using a filtration manifold, and mounting
229 filters on a microscope slide for enumeration. These procedures are detailed below; note that a

230 centrifugation based method (described in Section 2.5) was subsequently developed to enable
231 processing of larger sample quantities.

232 Each candidate probe was tested with the cultures listed in Table 2 to verify specificity
233 and efficacy. For this initial cross-reactivity testing, 300 μl of formalin-methanol preserved
234 culture was loaded into each chamber of a hybridization manifold. Methanol was removed via
235 vacuum filtration, and particulates were collected on a Cyclopore membrane (5 μm pore size,
236 25mm diameter; Whatman, NJ, USA). Samples were treated with prehybridization buffer (5X
237 SET, 0.1% IGEPAL CA-630, Poly A 10 mg mL^{-1}, 10% formamide) and incubated at room
238 temperature for five minutes. Prehybridization buffer was removed via vacuum filtration and
239 replaced with hybridization buffer containing FISH probes at a concentration of 2 ng μl^{-1}.
240 During all protocol steps, exposure of probes to light was minimized in order to prevent
241 fluorophore quenching. Samples were incubated in a hybridization manifold at 52, 53, or 55 °C
242 for an hour, depending on the Tm of the probe mixture. Hybridization buffer was removed via
243 vacuum filtration and 0.2X SET wash solution was added and incubated for five minutes at room
244 temperature. The wash solution was removed via vacuum filtration, and hybridization filters
245 were placed on glass slides, mounted with 5-10 μl glycerol (depending on sample density), and a
246 glass cover slip applied. Slides were stored in the dark at 4°C until microscopic enumeration
247 using fluorescence microscopy, and all analyses were completed within 72 hours of
248 hybridization to avoid loss of signal.

249 Following the initial culture-based testing for specificity, probes were then tested using
250 field samples from the Florida Keys (Heine Grass Bed, HGB: 24.859667; -80.73816) and St.
251 Thomas, USVI (Flat Cay, FC: 18.31822083; -64.99103593; Black Point, BP: 18.34417968; -
252 64.98543862) that were routinely collected as part of an ongoing study to monitor and model

253 *Gambierdiscus* abundance and toxicity (CiguaHAB). Two turf algae samples collected from the
254 Florida Keys in March 2018 and Feb 2019 (West Washer Woman: WWW: 24.5475; -81.5866;
255 Tennessee Reef Lighthouse, TRF: 24.745; -80.7812) were used in spiking experiments using
256 cultures of known to assess recovery and to confirm that *Gambierdiscus* can be successfully
257 quantified by FISH in the presence of detritus and other organisms. These samples had been
258 previously enumerated and were known to be devoid of *Gambierdiscus*. A description of sample
259 collection and processing procedures can be found in Parsons et al. (2017).
260 Macroalgal samples from St. Thomas were first used in qualitative testing. These samples
261 were characterized by high amounts of detritus, allowing testing of probe efficacy in the
262 presence of potentially confounding environmental particulates. For this analysis, samples were
263 spiked with known *Gambierdiscus* species from culture (~0.5 culture per 14 mL sample); these
264 spiked samples were used to determine whether probe concentrations were sufficient to label
265 cells when they were a minor fraction of a larger benthic community (as determined from
266 preliminary data collected over several years at these locations). Hybridizations were performed
267 as described above using 1 mL of sample. Probes were tested at 2 ng μl^{-1}, 3 ng μl^{-1}, and 4 ng μl^{-1}
268 in each hybridization reaction using the Cy3 fluorophore, and 1.3 µl mL^{-1} of a 10 mg mL^{-1}
269 working stock solution of Calcofluor White M2R was added to the SET wash solution. As cells
270 were easily visualized at a probe concentration of 2 ng μl^{-1}, this was selected as the working
271 concentration for further analysis.
272
273 2.5. *Quantitative assessment of FISH approach for Gambierdiscus cell enumeration*
274 In order to confirm that *Gambierdiscus* can be successfully quantified by FISH in the
275 presence of detritus and other organisms, a series of experiments were performed in which field

276 samples were spiked with known concentrations of *Gambierdiscus* cultures, and these samples
277 were enumerated using FISH. For these experiments, cultures of *G. silvae, G. carolinianus,* and
278 *G. caribaeus* were preserved as described in Section 2.3. Cell concentrations of each were first
279 determined by staining 1 mL of sample with Calcafluor White (n=6), and these samples were
280 enumerated at 100× using a DAPI filter.

281 Two turf algae samples from sites in the Florida Keys (WWW & TRF) which had been
282 previously identified as being devoid of *Gambierdiscus spp*. based on prior sample enumeration
283 were pooled and used for these experiments. Each methanol-preserved field sample was spiked
284 with 1 mL of culture (n=3), hybridized with the corresponding species-specific probe as
285 described below, and enumerated.

286 For this experiment, a modified centrifugation method was used for hybridization rather
287 than the filtration manifold approach described previously. In this modified protocol, samples
288 were centrifuged (5 min x 10000 *g*) to pellet contents and overlying methanol was aspirated,
289 taking care to leave the pellet undisturbed. The pellet was resuspended in 1 mL hybridization
290 buffer (5X SET, 0.1% IGEPAL CA-630, Poly A 10 mg mL^{-1}, 10% formamide) and incubated at
291 room temperature for five minutes. Probe was added at a final concentration of 1.6 ng μl^{-1}, and
292 samples were incubated in the dark at 53 or 55°C (Tm-dependent) for one hour. Following the
293 incubation, samples were pelleted via centrifugation, and hybridization buffer was aspirated and
294 replaced with 1 mL wash buffer (0.2X SET solution). Samples were incubated at room
295 temperature for five minutes and centrifuged. Wash buffer was aspirated and pellet was
296 resuspended in 1 mL of 5X SET solution containing 10 µl of a working stock solution of
297 Calcafluor White (10 mg mL^{-1}), and 1 mL of sample was loaded into a Sedgewick-Rafter slide
298 for identification and enumeration under fluorescence. This modified centrifugation method has

299 proven effective for processing large numbers of samples, and quality of labeling is comparable
300 to the manifold filtration method.
301 As described in Section 2.1, each slide was initially scanned under the DAPI filter set.
302 Whenever a *Gambierdiscus* cell was identified (to genus), the Cy3, Texas Red, and FITC filters
303 were applied in sequence in order to determine whether the cell was labeled with a fluorophore.
304 The filter set was then returned to DAPI, and scanning continued until another *Gambierdiscus*
305 cell was located. Differences in cell concentrations determined by microscopic counts versus
306 FISH enumeration were compared using a non-parametric Wilcoxon test performed using JMP
307 11 (SAS Corporation, NC, USA).
308
309 2.6. *Field Sample Testing*
310 In the Caribbean, field samples collected from the HGB site within the Florida Keys
311 (Parsons et al., 2017) were selected for a seasonal comparison of *Gambierdiscus* community
312 diversity. These samples were collected from two macroalgal hosts (*Thalassia testudinum* and
313 *Halimeda incrassata*) from June to December in 2013. As these samples were analyzed prior to
314 development of the multiplexing approach, individual probes detecting *G. belizeanus, G.*
315 *caribaeus*, *G. carolinianus, G. carpenteri*, and the *G. silvae/G. polynesiensis* clade were used,
316 each attached to the Cy3 fluorophore. Multiple hybridizations carried out for each sample as
317 described in Section 2.4, using a probe concentration of 2 ng μl^{-1}. In the Pacific, field samples
318 were collected from sites within the Wai'Ōpae Tide Pools on the southeastern shore of the Island
319 of Hawai'i (19.487994, -154.821981) in July 2015 to determine probe efficacy in this region.
320 Macroalgae were scarce in this environment, so artificial substrates (e.g., window screens) were
321 used in sampling to provide representative sampling of the benthic dinoflagellate community.

322 Artificial substrates were deployed for 24 hours at several locations in this system over the
323 course of one week and processed following established methods (Tester et al., 2014). As in
324 previous studies, screen surface area for *Gambierdiscus* abundance measurements was calculated
325 by considering the screen to be composed of cylindrical filaments resulting in a total surface area
326 of 156.74 cm^2 (Tester et al., 2014). These samples were analyzed with the Pacific assay probes
327 (Table 1), following the multiplexing approach. This testing illustrated the efficacy of the
328 Pacific probes as well as multiplexing with combinations of three and two probes.
329

330 **3. Results**

331

332 *3.1. Probe design*

333 Molecular probes were designed for a range of *Gambierdiscus* species and clades as
334 shown in Figure 1, based on their common occurrence at our sampling sites in St. Thomas, USVI
335 and the Florida Keys, as documented in the scientific literature (Litaker et al., 2010; Lozano-
336 Duque et al., 2018), and depending on the availability of cultured isolates that could be used in
337 testing. Therefore, not all species were addressed in this effort, including *G. scabrosus*, *G.*
338 *balechii*, *G. toxicus*, *G. excentricus* and *G. pacificus*. Molecular probe sequences are shown in
339 Table 1.
340

341 *3.2 Cross-reactivity testing using Gambierdiscus cultures*

342 Species-specific candidate probes labeled with Cy3 fluorophores were tested using
343 cultured isolates of *Gambierdiscus* (Tables 2 and 3) to establish specificity and identify cross-

344 reactivity (Table 3, Fig. 2). Determination of labeling specificity was based on visual intensity of
345 fluorescence in comparison with non-target cells (Fig. 2).
346 Probes Gaust_D1D3, Gcarib_D1D3, Gbeliz_D8D10, Gcarpent_D1D3, and
347 Gcarol_D8D10 only detected their target species (Fig. 2) but probes Gpoly_D8D10 and
348 Gribo1_D8D10 detected both *G. polynesiensis* and *G. silvae*. For all known ribosomal large
349 subunit sequences both Gpoly_D8D10 and Gribo1_D8D10 have at least two base pairs of
350 difference between the species; these species are very closely related, however (Fig. 1). The
351 clade comprising *G. polynesiensis* and *G. silvae* can potentially be labeled by both probes
352 Gpoly_D8D10 and Gribo1_D8D10 (the species *G. carolinianus* was not labeled by these
353 probes); therefore, for this and future analyses, probes Gpoly_D8D10 and Gribo1_D8D10 have
354 been redefined to detect species within the highly toxic "*G. polynesiensis* clade".
355
356 *3.3 Multiplexing fluorophores to detect multiple species*
357 Multiplexed probe assays for each geographic region were created based on optimal Tm
358 for probe hybridizations (Tables 1 and 3). Up to three probes were multiplexed via differential
359 labeling by multiple fluorophores (See Table 1, Fig. 3). These fluorophores were chosen based
360 on labeling efficacy and their ability to be combined due to their emission of light in separate
361 spectral regions, and allows multiple species in a single sample to be distinguished and
362 enumerated.
363 The use of Calcafluor White to stain cells allows for initial identification of
364 *Gambierdiscus* at the genus level when field samples are scanned under the DAPI filter set.
365 While other members of the algal community, such as *Ostreopsis* and *Prorocentrum*, are also
366 stained by Calcafluor White, their morphology is readily distinguishable from *Gambierdiscus*

367 based on thecal plate structure and other cellular characteristics. The Calcafluor stained cells are
368 also readily distinguished from background detritus. Once cells were identified at the genus
369 level, species-level identification was possibly based on determined by overall brightness,
370 relative intensity, and color of the cell when visualized under the three filter sets (Fig. 3). For
371 example, cells labeled with Cy3 (or Alexa Fluor® 532) were brightly labeled and orange in
372 appearance under the Cy3 filter set; these cells are visible but dark and faded under the FITC and
373 Texas red filters. Cells labeled with the Texas Red fluorophore (or Alexa Fluor® 594) appeared
374 brightly lit and vibrantly red under the Texas Red filter, but appeared as faded red under Cy3
375 filter, and barely visible under the FITC filter. Cells labeled with FITC (or Alexa Fluor® 488)
376 were a bright and chalky green under the FITC filter, and barely visible under the other two
377 filters. Unlabeled cells are only faintly visible under all three filter sets. Labeling can be thus be
378 distinguished by a combination of color intensity and brightness, and for the multiplexing
379 approach to be effective, it is important to carefully visualize each cell under all three filter sets.
380 A note regarding autofluorescence: this can introduce interference if field samples are
381 not properly preserved (Section 2.3). High levels of autofluorescence (Fig. 4) will manifest as a
382 bright appearance under all three filter sets, complicating cell identification. In our experience
383 autofluorescence is minimized by following preserving samples promptly, and that delays in
384 preservation of over 24 hours introduced by occasional logistical disruptions to field operations
385 produced samples with higher autofluorescence.
386
387 3.4 *Quantitative Assessment of Gambierdiscus species enumeration using FISH*
388 Experiments were performed to confirm that FISH allows quantitative enumeration of
389 *Gambierdiscus* species in the community matrix typically present in field samples (e.g., detritus

390 and other organisms). Field samples collected from the Florida Keys spiked with *Gambierdiscus*
391 cultures yielded reliable labeling and detection of cells (Table 4). No significant differences in
392 cell density were found between cultures and spiked field samples for any of the three species
393 tested (*G. caribaeus,* p=0.52; *G. carolinianus,* p=1; *G. silvae*, p=0.25). These results indicate that
394 the FISH method results in robust quantification of *Gambierdiscus* cells in field samples, and
395 that the processing steps involved do not result in significant cell loss. Low variability among
396 replicate samples also demonstrates that the centrifugation method is a viable alternative to the
397 manifold filtration method. This latter approach also allows for processing of larger sample
398 volumes; this is highly advantageous when enumerating *Gambierdiscus* spp., which are often
399 present at low concentrations and are frequently a minor component of the benthic dinoflagellate
400 assemblage (e.g., Richlen and Lobel, 2007).
401
402 *3.5. Field Sample Analysis*
403 To determine the efficacy of *Gambierdiscus* cell detection in field samples, benthic
404 samples collected from the HGB site in the Florida Keys were labeled with individual probes
405 detecting *G. belizeanus, G. caribaeus*, *G. carolinianus, G. carpenteri*, and the *G. polynesiensis*
406 clade. These analyses showed that the *Gambierdiscus* community primarily consisted of the *G.*
407 *polynesiensis* clade, *G. caribaeus,* and *G. carpenteri; G. carolinianus* was a minor component of
408 the *Gambierdiscus* community, and *G. belizeanus* was absent (Fig. 5). The cells in the *G.*
409 *polynesiensis* clade were likely *G silvae*, as *G. polynesiensis* has not been reported in the
410 Caribbean (Litaker et al., 2010; Lozano-Duque et al., 2018).
411 The *Gambierdiscus* community composition shifted over time as total *Gambierdiscus*
412 spp. abundance increased, predominantly during November and December. All species detected

413 were at low concentrations (<5 cells cm^{-2}) from the outset of the sampling in June until

414 November when three of the four species present increased (Fig. 5). *Gambierdiscus caribaeus*

415 and *G silvae* become the dominant taxa in November, whereas in December, the two dominant

416 species were *G. caribaeus* and *G. carpenteri*. Overall *Gambierdiscus* genus abundance also

417 increased in the fall and early winter portion of the sampling period.

418 To test probes specifically designed for Pacific *Gambierdiscus* species and to determine

419 efficacy of probe multiplexing (using multiple fluorophores), field samples collected in 2015

420 from Wai'Ōpae Tide Pools, Hawai'i were labeled with probes detecting *G. australes, G.*

421 *belizeanus, G. caribaeus*, *G. carpenteri*, and the *G. polynesiensis* clade (Table 1). All species

422 were readily detected at the site with these specific probes and fluorophores. Operationally,

423 using combinations of 2-3 fluorophores per sample permitted visualization of up to three species

424 at once using the three filter sets. Variation in species composition was observed between sites

425 and pools across Wai'Opae during a single week-long sampling period (Fig. 6). There was a

426 large proportion of *G. australes* present overall with *G. caribaeus* and *G. belizeanus* also

427 proportionally the most abundant in sites 2 and 6, respectively. The highest concentration of

428 *Gambierdiscus* cells was found in the sites furthest from shore.

429

430 **4. Discussion**

431 Recent advances in research on the taxonomy and toxicity of *Gambierdiscus* spp. have

432 demonstrated that multiple species with widely differing toxicities co-occur within a particular

433 locale and even on the same host alga (e.g., Vandersea et al., 2012; Nishimura et al., 2016), yet

434 the cryptic diversity of this genus precludes identification beyond the genus level when

435 analyzing samples using traditional light microscopy. Some dinoflagellate and diatom HAB

436 genera exhibit variability in toxin content among species and strains (e.g., *Alexandrium*, *Pseudo-*
437 *nitzschia*; Maranda et al., 1985; Cembella et al., 1987; Bates et al., 1998), a trait that has also
438 been documented in *Gambierdiscus* (Chinain et al. 1999; 2010, Litaker et al., 2017; Robertson et
439 al. 2018, Longo et al. 2019). It is therefore likely that *Gambierdiscus* communities at a given
440 location are comprised of both highly toxic and non-toxic (or low toxicity) species, making
441 species-specific enumeration a critical component in determining risk of ciguatera fish
442 poisoning. The development of species-specific fluorescence *in situ* hybridization (FISH) probes
443 allows for accurate enumeration of *Gambierdiscus* species in field samples, and offers a
444 quantitative method for determining community composition in field samples, and particularly
445 for monitoring the toxic species *G. silvae* and *G. polynesiensis*. As additional species-specific
446 PCR primers and probes become available, the approach outlined herein can also be adapted for
447 additional species and clades, including other toxic species of interest (e.g., *G. excentricus*).
448 Fluorescence *in situ* hybridization has been adapted for enumeration of several harmful
449 algal bloom (HAB) organisms worldwide, including toxin-producing *Alexandrium catenella*
450 (Anderson et al., 2005; John et al., 2005), *Prorocentrum micans* (Chen et al., 2013), *Pseudo-*
451 *nitzschia* spp. (Greenfield et al., 2006; Parsons et al., 1999; Scholin et al., 1996), and the fish
452 killing raphidophyte *Heterosigma akashiwo* (Chen et al., 2008). These species are sometimes
453 present in relatively low proportions compared to the rest of the planktonic community, and
454 identification may be further confounded by morphological similarities to other more innocuous
455 organisms. The FISH method enables quick and high-confidence visual identification of toxic
456 taxa. Given the potential for variable toxicity among *Gambierdiscus* species and strains, species
457 composition may have a greater impact on risk of CFP than total genus abundance. The latter is,
458 however, the parameter typically reported in field studies related to CFP. As a result of this

459 study, probes can be targeted towards specific *Gambierdiscus* species or groups, allowing
460 selective enumeration of species known to exhibit high toxicity. An example of the magnitude of
461 this effect is seen in Figure 5. In mid-November, *G. caribaeus* was the dominant taxa, with
462 roughly twice the abundance of the *G. polynesiensis* clade (presumably *G. silvae*), yet the latter
463 is several orders of magnitude more toxic (Robertson et al., 2018), and is the most likely source
464 of ciguatoxins in fish at this location based on our prior characterization of *Gambierdiscus*
465 community composition and structure in St. Thomas and the Florida Keys (Lozano-Duque et al.,
466 2018). Even when *G. silvae* concentrations decreased in December as *G. caribaeus* and *G.*
467 *carpenteri* increased, the vast majority of the ciguatoxin at that location would still be
468 attributable to *G. silvae*.

469 Using FISH, species abundance measurements could be integrated into routine
470 monitoring efforts and analyzed over spatiotemporal gradients, allowing establishment of
471 species-specific seasonal patterns and elucidation of community responses to disturbances, such
472 as storms or coral bleaching. Preference for substrates or other environmental niche parameters
473 can also be determined via experimental manipulations in the field or in the laboratory, followed
474 by species-specific FISH probe analyses. In this context, note that in the field samples analyzed
475 for this study, discrepancies were observed between the total abundance of *Gambierdiscus* cells
476 and the summed abundance of all species detected using the FISH probes (Figs. 5, 6). One
477 possible explanation is the presence of unlabeled cells or incomplete labeling, but this could also
478 reflect the presence of one or more known or unknown species for which there is currently no
479 FISH probe developed, and is a fertile area for further investigation. As knowledge of
480 *Gambierdiscus* species taxonomy expands (e.g., Fraga and Rodríguez, 2014) the discovery of
481 new species will force re-evaluation of detection techniques. The FISH method can be readily be

482 adapted to target new species of interest. An additional benefit of this method is that it can be
483 used to interrogate archived field samples preserved in methanol, allowing retrospective analysis
484 of samples that had previously been used for genus or species enumeration.

485 Current methods for species-level *Gambierdiscus* detection such as electron micrograph
486 imaging or Sanger DNA sequencing of cultures require extensive time and effort, which can be
487 prohibitive for routine monitoring. Another approach that has shown promise is the use of a
488 semi-quantitative qPCR assay to detect species presence in field samples (Vandersea et al.,
489 2012). Through DNA extraction, the qPCR assay can examine a greater volume of sample more
490 rapidly than FISH analysis. However, this technique may be subject to matrix effects associated
491 with algal host substrate, which can impact limits of detection. The FISH approach described in
492 this study allows for quantitative enumeration of all types of field samples in a time-efficient
493 framework. Integration of multiple methods is likely to be an effective approach, as suggested in
494 Kibler et al. (2015). For example, qPCR could be used to determine presence-absence or the
495 relative abundance of an array of species (e.g., Nishimura et al., 2016; Vandersea et al., 2012)
496 and then FISH probes could be used to the abundance of certain select taxa.

497 In this regard, accurate identification is essential to establishing patterns of abundance in
498 field samples, and ultimately predicting future blooms. To date, predictive modeling efforts for
499 *Gambierdiscus* have been limited to the genus level (Parsons et al., 2010) and are not yet able to
500 forecast CFP risks. Field studies have been inconsistent in establishing a positive relationship
501 between toxicity and cell abundance, or with environmental factors such as ocean warming
502 (Chateau-Degat et al., 2005, Radke et al., 2013). To resolve these questions, spatiotemporal
503 dynamics of individual species, quantitatively measured by FISH probe analysis, can be
504 integrated with environmental data to update and refine models and analyses of trends. Measured

505 abundances can be correlated with parameters such as temperature, nutrient levels, light, salinity,
506 wave action, and season. Species-specific growth and toxicity data under various treatments (Xu
507 et al., 2012, Kibler et al., 2014) can be used to inform new models, with the ultimate goal of
508 improving CFP predictive abilities.
509

510 **5. Conclusions**

511 Species-specific FISH probes are a powerful new tool enabling those working with field
512 populations of *Gambierdiscus* species to investigate community structure, and monitor the
513 abundances of the most toxic, and thus the most important taxa. Due to *Gambierdiscus* species'
514 cryptic diversity and variance in toxicity, species-specific enumeration is a critical component in
515 the determination of regional risk of ciguatera fish poisoning. This novel approach was
516 successfully applied to samples from both the Caribbean Sea and Pacific Ocean, demonstrating
517 high specificity in field samples and cultures from both regions. Moving forward, this method
518 will facilitate the creation of datasets with high resolution at the species level. Analysis of
519 *Gambierdiscus* community composition across multiple environments and over time will allow
520 species abundance to be linked to environmental parameters, improving our ability to understand
521 and manage the current and changing risks of CFP worldwide.
522

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533

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684 **Table 1**. Probe combinations for the analysis of samples from the Caribbean and Gulf of Mexico, and
685 Pacific Ocean. Table shows probe target species, sequence, melting temperature (Tm),
686 microscope filter, and fluorophore used for illumination. LP=long pass; BP=band pass.
687

CARIBBEAN SEA REGION PROBE COMBINATIONS						
Group	**Probe**	**Target Species**	**Sequence**	**Tm**	**Filter**	**Fluorophore**
1	Gcarib_D1D3	*G. caribaeus*	TGAGACCCACATGTGGAGATTC	**53**	FITC (LP, Zeiss 09)	AF488
	Gbeliz_D8D10	*G. belizeanus*	AGATCAGTACGCCAGAGTGACTA		Cy3 (LP, Chroma 49016)	AF532
2	Gcarpent_D1D3	*G. carpenteri*	TGATGTAACGCAAGACGCACAG	**55**	FITC (LP, Zeiss 09)	AF488
	Gribo1_D8D10	*G. polynesiensis* clade	CGATCAGAGACATACTTTGGCGC		Cy3 (LP, Chroma 49016)	AF532 or Cy3
	Gcarol_D8D10	*G. carolinianus*	AGCAAGCCACAGATCCACTGAG		TxRd (LP, Chroma 19006	AF594
PACIFIC OCEAN PROBE COMBINATIONS						
Group	**Probe**	**Target Species**	**Sequence**	**Tm**	**Filter**	**Fluorophore**
1	Gcarib_D1D3	*G. caribaeus*	TGAGACCCACATGTGGAGATTC	**52**	FITC (LP, Zeiss 09)	AF488
	Gaust_D1D3	*G. australes*	TGCCAATCCAGTTGTGTATCTC		Cy3 (LP, Chroma 49016)	AF532
2	Gcarpent_D1D3	*G. carpenteri*	TGATGTAACGCAAGACGCACAG	**53**	FITC (LP, Zeiss 09)	AF488
	Gbeliz_D8D10	*G. belizeanus*	AGATCAGTACGCCAGAGTGACTA		Cy3 (LP, Chroma 49016)	AF532 or Cy3
	Gpoly_D8D10	*G. polynesiensis* clade	CTCCGCCAGTGACGTTAAGTAG		TxRd (LP, Chroma 19006)	AF594

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Table 2. Cultured isolates of *Gambierdiscus* spp. used for candidate probe testing. Conditions for culture maintenance are described in Xu et al. (2014).

Isolates	**Geographic Origin**	**Species**
CCMP 1653	Hawaiʻi, USA	*G. australes*
CCMP 399	St. Barthélemy Island, French West Indies	*G. belizeanus*
GTBNAC1	Florida Keys, FL, USA	*G. caribaeus*
BP Aug08	St. Thomas, USVI, USA	*G. caribaeus*
BB Apr10_6	St. Thomas, USVI, USA	*G. carolinianus*
BP Mar10_1	St. Thomas, USVI, USA	*G. carolinianus*
HGB6	Florida Keys, FL, USA	*G. carpenteri*
350509-27	Marakei, Republic of Kiribati	*G. pacificus*
D50511-08	Marakei, Republic of Kiribati	*G. pacificus*
RIK-8	Mangareva, Gambier, French Polynesia	*G. polynesiensis*
RAI-1	Raivavae, Australes, French Polynesia	*G. polynesiensis*
RG-92	Rangiroa, Tuamotu, French Polynesia	*G. polynesiensis*
SH Apr11-1	St. Thomas, USVI, USA	*G. silvae*
TRL23	Florida Keys, FL, USA	*G. silvae*
FC May10_9	St. Thomas, USVI, USA	*G. silvae*
BP Mar10_5	St. Thomas, USVI, USA	*Gambierdiscus* sp. *ribotype 2*

1 Table 3. Results of cross-reactivity using cultured isolates of *Gambierdiscus*. Cultures were preserved in formalin and methanol,
2 hybridized with a Cy3-labeled probe, and viewed under Cy3 filter using fluorescence microscopy. Probes that successfully
3 hybridized with an isolate and were highly visible under the fluorescent microscope are denoted positive "+"'Pos'. Probes that
4 failed to detect a culture are denoted negative "-". Probes were tested at all Tms listed in Table 1.

		Probe/Target Species						
Culture ID	**Species**	Gaust_D1D3/ *G. australes*	Gcarib_D1D3/ *G. caribaeus*	Gbeliz_D8D10/ *G. belizeanus*	Gpoly_D8D10/ *G. polynesiensis* clade	Gcarpent_D1D/ *G. carpenteri*	Gcarol_D8D10/ *G. carolinianus*	Gribo1_D8D10/ *G. polynesiensis* clade
CCMP 1653	*G. australes*	+	-	-	-	-	-	-
CCMP 399	*G. belizeanus*	-	-	+	-	-	-	-
BP Apr 11_7	*G. belizeanus*	-	-	+	-	-	-	-
BP Mar 10_6	*G. belizeanus*	-	-	+	-	-	-	-
GTBNAC1	*G. caribaeus*	-	+	-	-	-	-	-
BP Aug08	*G. caribaeus*	-	+	-	-	-	-	-
BB Apr10_6	*G. carolinianus*	-	-	-	-	-	+	-
BP May10_1	*G. carolinianus*	-	-	-	-	-	+	-
HGB6	*G. carpenteri*	-	-	-	-	+	-	-
RIK-8	*G. carpenteri*	-	-	-	-	+	-	-

RAI-1	*G. polynesiensis*	-	-	-	+	-	-	+
RG-92	*G. polynesiensis*	-	-	-	+	-	-	+
350509_271	*G. pacificus*	-	-	-	-	-	-	-
D50511-08	*G. pacificus*	-	-	-	-	-	-	-
SH Apr11-1	*G. silvae*	-	-	-	+	-	-	+
TRL23	*G. silvae*	-	-	-	+	-	-	+
FC May 10_9	*G. silvae*	-	-	-	+	-	-	+
BP Mar 10_5	*G.* ribotype 2	-	-	-	-	-	-	-

Table 4. Quantitative assessment of FISH approach for *Gambierdiscus* cell enumeration. Cultures of *G. silvae*, *G. carolinianus*, and *G. caribaeus* were enumerated using microscopic cell counts, and known concentrations added to field samples. These samples were analyzed using FISH with corresponding species-specific probes.

Species	Cell abundance microscopic cell counts (1 mL, n=6)	Cell abundance FISH (1 mL, n=3)
G. carolinianus	66±4.5	67±9.3
G. silvae	54±8.9	72±2.7
G. caribaeus	42±6.2	51±8.7

Figure Captions

Figure 1. Phylogeny based on analysis of the D8-D10 region of LSU rRNA gene of *Gambierdiscus* species, adapted from Xu et al. (2014). Scale bar = 0.05 substitutions per site. Support values are Bayesian posterior probability and bootstrap support values from maximum likelihood analysis. In orange is highlighted the '*Gambierdiscus polynesiensis* clade'. In green, species for which this study has developed species-specific probes are highlighted.

Figure 2. Fluorophore labels targeted to five species (*G. caribaeus, G. belizeanus, G. carpenteri, G. carolinianus, G. silvae*) hybridized across seven different cultures. Strain-specific information for each culture is indicated to the right. During initial testing, cultures were hybridized in

separate reactions within a hybridization manifold with individual candidate probes labeled with either a Cy3 or FITC fluorophore, which were cost-effective for candidate probe screening.

Figure 3. Fluorophore labeling and identification of *Gambierdiscus* cells hybridized to species-specific probes using multiplexed fluorophores. Each row of micrographs displays a single *Gambierdiscus* cell under transmitted light followed by DAPI, Cy3, Texas Red and FITC filters. The species and labeling of each cell are indicated on the left-hand side of the figure. The final row of micrographs displays an unlabeled cell. The use of Calcofluor White allows visualization of thecal plates under DAPI filter, and also aids in distinguishing *Gambierdiscus* cells from surrounding detritus.

Figure 4. Demonstration of autofluorescence variability in field samples caused by improper sample preservation. Each row of micrographs displays a single unlabeled *Gambierdiscus* cell viewed under transmitted light followed by DAPI, Cy3, Texas Red and FITC filters. The top row displays a highly autofluorescent cell from a sample that was stored for over 24 hours before formalin-methanol fixation, and the bottom row shows a minimally autofluorescent cell from a field sample that was preserved properly (see Section 2.3 for preservation protocol).

Figure 5. Community dynamics of *Gambierdiscus* species on *Halimeda* macroalgae at the Heine Grassbed (HGB) site over a six-month period. Species composition is similar to that observed in other studies in the Caribbean. Total abundance of *Gambierdiscus* spp. (genus level count) is shown by the dashed black line and summed abundance of enumerated species is shown by the dashed grey line, illustrating the fraction of unidentified cells. *Gambierdiscus*

species detections shown by colored lines. Pie charts show relative species composition at each sampling point.

Figure 6. *Gambierdiscus* community at Wai'Ōpae Tide Pools on the southeastern shore of the Island of Hawai'i during July 2015 as taken from deployed artificial substrates. (A) Tide pool boundaries are outlined in orange with sampling location and sampling site number indicated. Pie charts show relative *Gambierdiscus* species abundance at each site. (B) *Gambierdiscus* cells per $100cm^2$ of artificial substrate for each site. Colored portions of bars show abundance of each species per site as shown in (A), and horizontal line and number indicates total abundance including unlabeled cells at each site.

Figure 1.

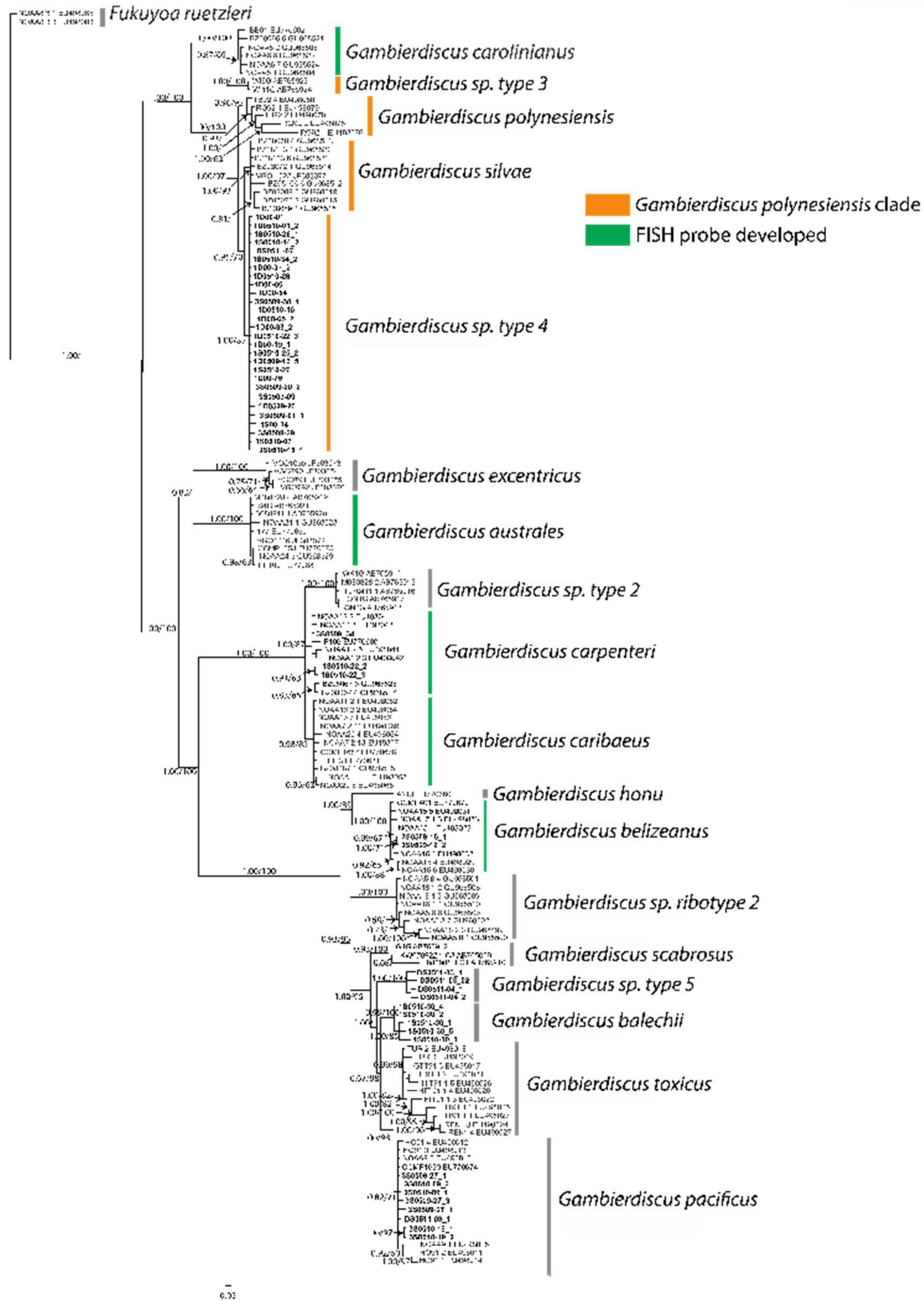


Figure 2.

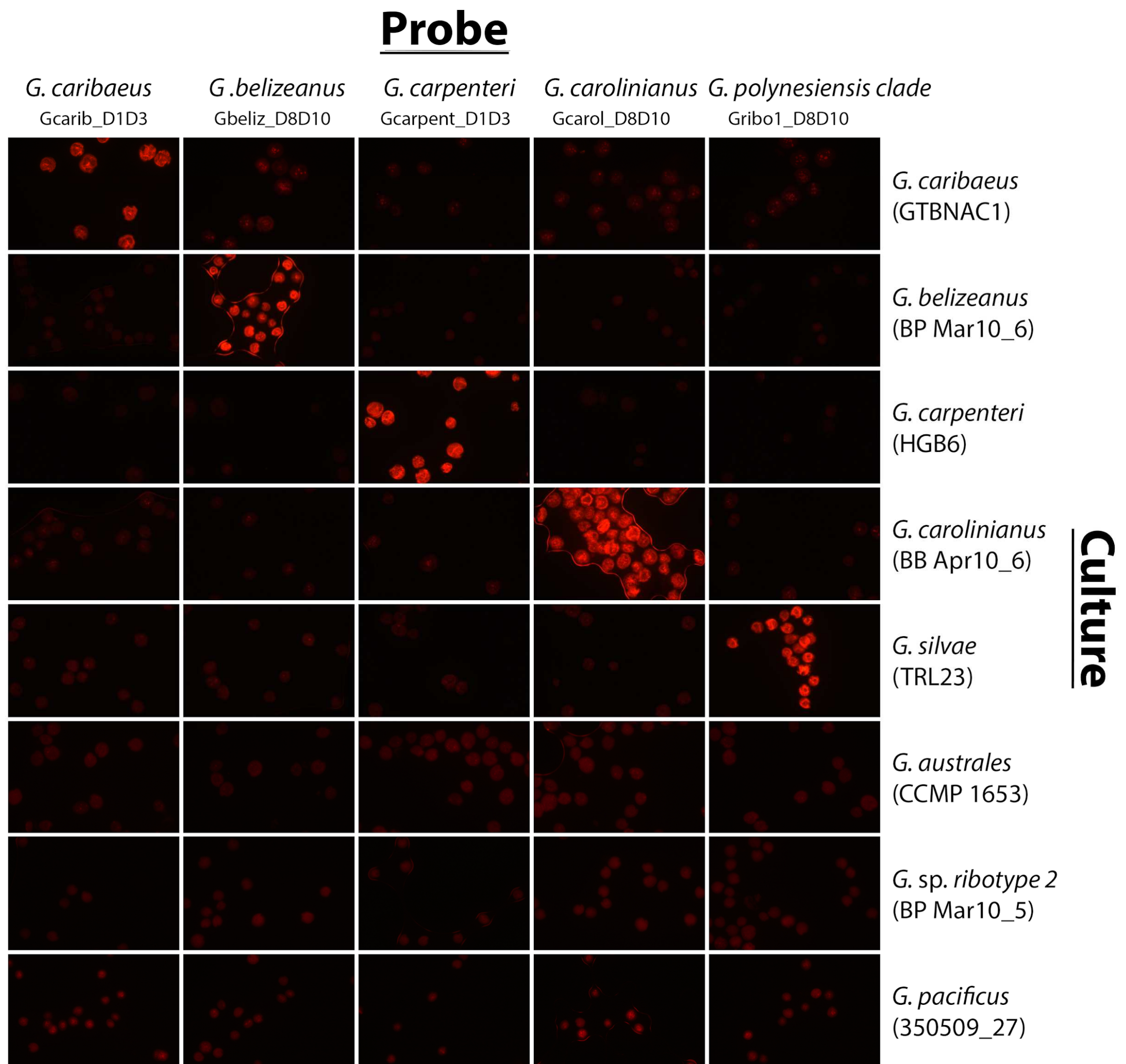


Figure 3.

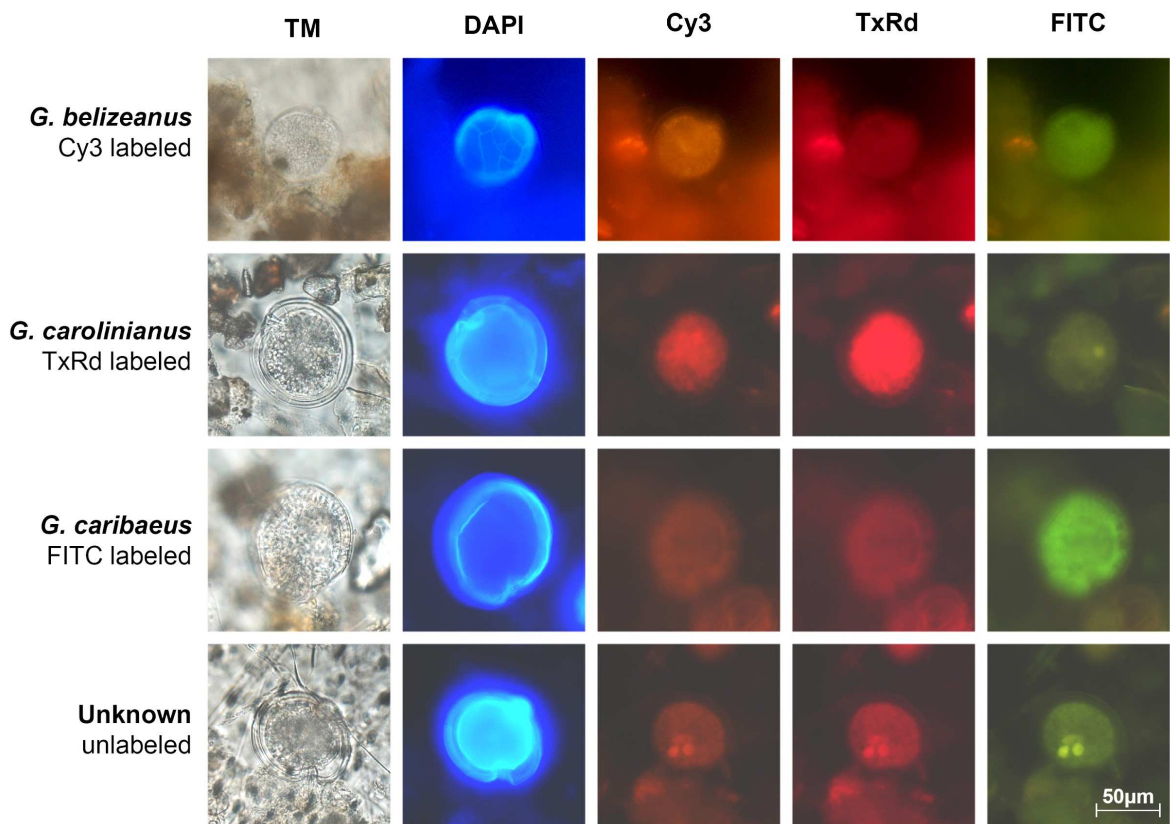


Figure 4.

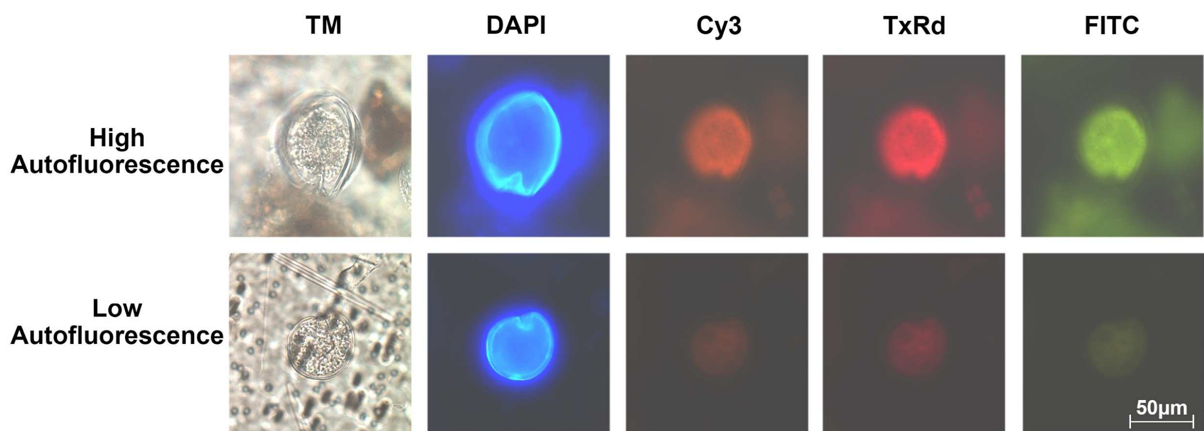


Figure 5.

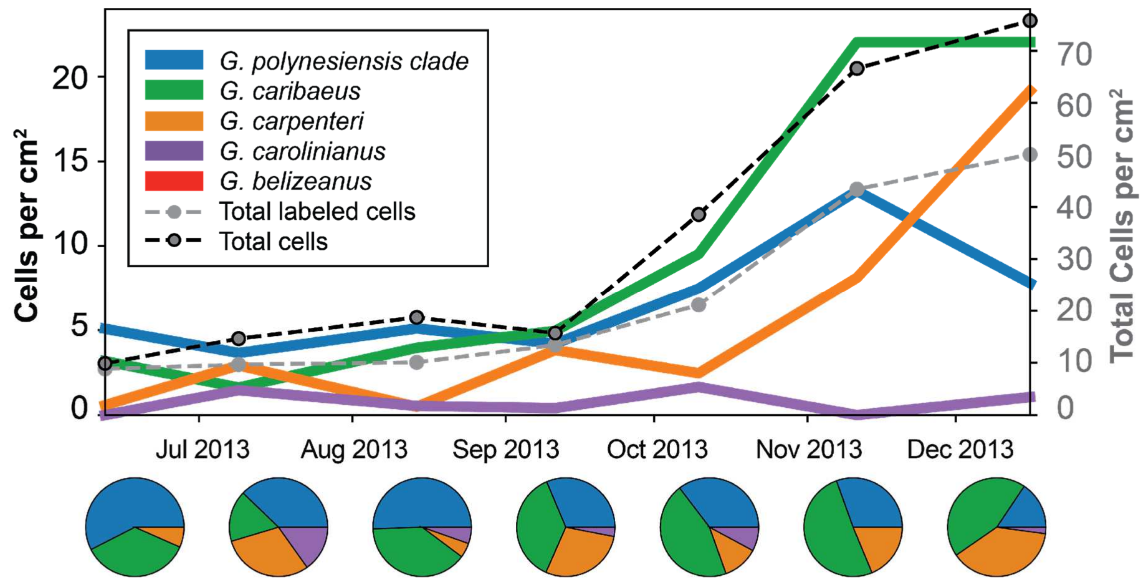
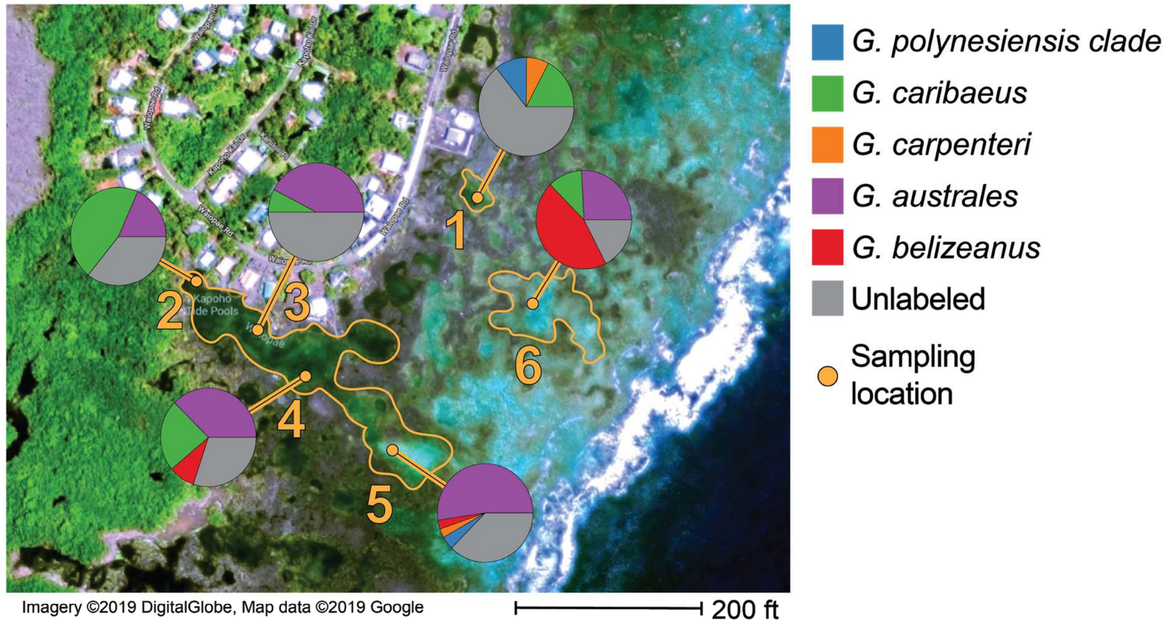
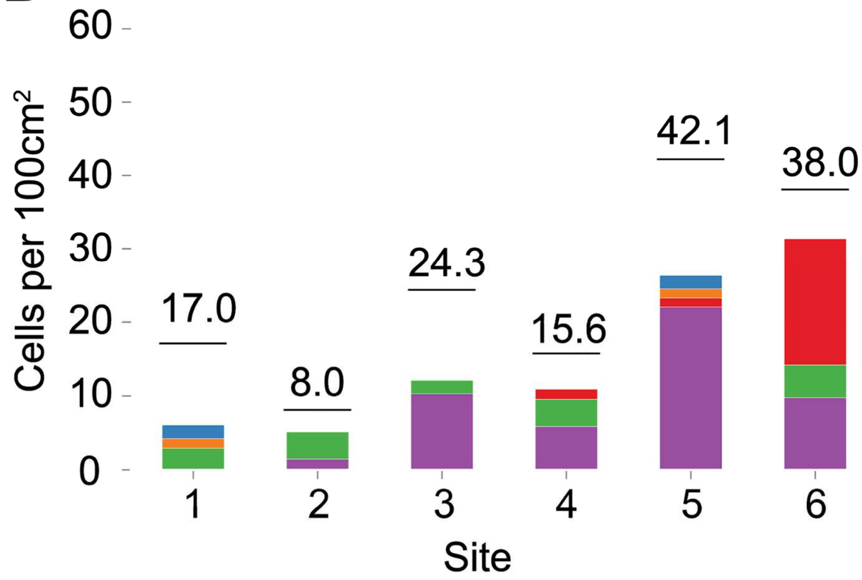


Figure 6.





Gambierdiscus sampled from substrate

FISH probes