1   The Prevalence of Benthic Dinoflagellates Associated with Ciguatera Fish Poisoning in

2   the Central Red Sea

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23 **Highlights**

24 - Macroalgae samples were collected from the central Red Sea and examined for the presence of

25 *Gambierdiscus* and *Ostreopsis*

26 - Both genera were observed at low densities, and significant differences in abundance were

27 detected between the two genera, and among sampling sites

28 - Multiple isolates established from field samples were morphologically and molecularly

29 identified as *Gambierdiscus belizeanus*

30 - Toxicity analysis confirmed *G. belizeanus* as a ciguatoxin producer

31

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Abstract

This study confirms the presence of the toxigenic benthic dinoflagellates *Gambierdiscus belizeanus* and *Ostreopsis* spp. in the central Red Sea. To our knowledge, this is also the first report of these taxa in coastal waters of Saudi Arabia, indicating the potential occurrence of ciguatera fish poisoning (CFP) in that region. During field investigations carried out in 2012 and 2013, a total of 100 *Turbinaria* and *Halimeda* macroalgae samples were collected from coral reefs off the Saudi Arabian coast and examined for the presence of *Gambierdiscus* and *Ostreopsis*, two toxigenic dinoflagellate genera commonly observed in coral reef communities around the world. Both *Gambierdiscus* and *Ostreopsis* spp. were observed at low densities (< 200 cells g^{-1} wet weight algae). Cell densities of *Ostreopsis spp.* were significantly higher than *Gambierdiscus* spp. at most of the sampling sites, and abundances of both genera were negatively correlated with seawater salinity. To assess the potential for ciguatoxicity in this region, several *Gambierdiscus* isolates were established in culture and examined for species identity and toxicity. All isolates were morphologically and molecularly identified as *Gambierdiscus belizeanus*. Toxicity analysis of two isolates using the mouse neuroblastoma cell-based assay for ciguatoxins (CTX) confirmed *G. belizeanus* as a CTX producer, with a maximum toxin content of $6.50 \pm 1.14 \times 10^{-5}$ pg P-CTX-1 eq. $cell^{-1}$. Compared to *Gambierdiscus* isolates from other locations, these were low toxicity strains. The low *Gambierdiscus* densities observed along with their comparatively low toxin contents may explain why CFP is unidentified and unreported in this region. Nevertheless, the presence of these potentially toxigenic dinoflagellate species at multiple sites in the central Red Sea warrants future study on their possible effects on marine food webs and human health in this region.

1. Introduction

Globally, marine algal toxins account for greater than 60,000 human intoxications annually, with an overall mortality rate of 1.5% (Van Dolah, 2000). Ninety percent of harmful phytoplankton species are flagellates, notably dinoflagellates (Smayda, 1997), and their biotoxins are responsible for an array of human illnesses, often associated with seafood consumption. The leading non-bacterial illness associated with seafood consumption is ciguatera fish poisoning (CFP) (WHO, 2009).

Certain species and strains of *Gambierdiscus* dinoflagellates produce ciguatoxins (CTX) and gambiertoxins that are precursors to CTXs. CTX is a liphophilic neurotoxin, and the consumption of fish that have accumulated these toxins leads to CFP (see reviews by Anderson and Lobel, 1987; Lehane and Lewis, 2000; Dickey and Plakas, 2010). These ciguatoxin-producing dinoflagellates are macroalgal epiphytes and are thus consumed by herbivorous and omnivorous fishes during grazing. Due to their bioaccumulation in the food web, levels of ciguatoxin are highest in carnivorous fish, particularly piscivores. Estimates of CFP incidence are uncertain as the disease is under-reported and often misdiagnosed (Friedman *et al*., 2008); however, as many as 200,000–1,000,000 people may be affected annually (Fleming *et al*., 1998; HARRNESS, 2005).

Thus far *Gambierdiscus* is the only dinoflagellate genus that has been definitively linked to ciguatera; however, several other co-occurring tropical and sub-tropical epiphytic species belonging to the genera *Ostreopsis*, *Prorocentrum*, and *Amphidinium* also produce toxins. These genera are well represented in most established benthic dinoflagellate communities in regions such as the Caribbean Sea, Pacific Ocean, and Indian Ocean (ciguatera is present in all these regions [Lehane and Lewis, 2000; Dickey and Plakas, 2010]), but the prevalence or impact of their toxins in the coral reef food chain is not well-documented or understood.

From a biogeographical perspective, *Gambierdiscus* species tend to be found in relatively low densities throughout most tropical and subtropical regions where they occur. Average *Gambierdiscus* spp. cell densities are similar in both the Atlantic and Pacific regions, with a slightly higher frequency

84 of high density samples (i.e., >1,000 cells g^{-1} wet weight algae) in the Pacific compared with the

85 Atlantic (Litaker *et al.,* 2010). *Ostreopsis* spp. cell densities vary enormously among geographic

86 regions, although abundances observed in tropical regions are generally lower than in temperate

87 regions. For example, cell densities reported from tropical regions range from <100 to 57,000 cells g^{-1}

88 wet weight algae (Grzebyk *et al.,* 1994; Kohler and Kohler, 1992), whereas in temperate regions cell

89 densities as high as 8.54 x 10^6 cells g^{-1} wet weight algae have been reported (Cohu *et al*., 2013). A

90 variety of macroalgae have been reported to host significant numbers of *Gambierdiscus* and other

91 epiphytic dinoflagellates (Cruz-Rivera and Villareal, 2006), although these dinoflagellates are also

92 found to be free living in sediments and coral rubble (Hallegraeff, 1993; Suburova *et al*., 2013), and in

93 the water column (e.g., Mangialajo *et al.,* 2008).

94   Despite recent advances in characterizing the global distribution of *Gambierdiscus*, there is

95 limited information regarding the presence of *Gambierdiscus* and the incidence of CFP in the Red Sea.

96 A recent study was the first to record the presence of the genus in the Arabian Gulf and in the northern

97 Red Sea (Suburova *et al*., 2013), but little else is known regarding the diversity, distribution, and

98 toxicity of *Gambierdiscus* species in the main body of the Red Sea. Similarly, there is scant data

99 regarding toxin levels in fish or the incidence of CFP from the Red Sea region, and none of the few

100 potential cases have been confirmed to arise from Red Sea fish (Ruprecht *et al*., 2001; de Haro and

101 Valli, 2003). As ciguatera is frequently unreported or misdiagnosed even in areas where it is well-

102 known, it is possible that the incidence of CFP in this region has been overlooked. Notably, the major

103 species of fish caught by traditional fisheries in the Saudi Arabian Red Sea include grouper, snapper,

104 emperors, barracuda, jacks, trevallies, kingfish, and tuna (Jin *et al*., 2012), which are known to be

105 ciguatoxic elsewhere in the world. The Red Sea generally suffers from a lack of sustained and

106 intensive ecological research compared with areas such as the Caribbean (Berumen *et al*., 2013), so

107 CFP or CTX may be present but remains undocumented. The objectives of the present study were to:

108 1) determine whether ciguatera-associated dinoflagellates (e.g., *Gambierdiscus* spp. and *Ostreopsis*

109 spp.) are present in coral reefs in the central Red Sea, 2) carry out a preliminary assessment of their

110   abundances and distribution at selected sampling sites, and 3) characterize the taxonomy and toxicity

111   of *Gambierdiscus* species present at these sites.

112   **2. Material and Methods**

113   *2.1 Sampling locations*

114   Field studies were carried out in the central Red Sea, including locations off the coasts of Thuwal and

115   Al-Lith, Saudi Arabia. Samples were collected from six inshore reefs (Fig. 1): Al Fahal (22° 17.919' N,

116   38° 58.053' E), Um Al Kiethl (22° 9.611' N, 38° 56.435' E); Um Al Balam (22° 11.880' N, 38° 57.055'

117   E), Abu Shosha (22° 18.182' N 39° 02.892' E), Mangrove Reef (20° 10.984' N, 40° 10.448' E), and

118   Coast Guard Reef (20° 8.942' N 40° 14.499' E); and four offshore reefs: Qita Al-Kirsh (22° 25.681' N,

119   38° 59.773' E); Sh'ib Nazar (22° 19.630' N, 38° 51.440' E), Malathu Reef (19° 44.310' N, 39° 54.070'

120   E), and Marmar Reef (19° 50.254' N, 39° 55.281' E).

121   *2.2 Algae collection*

122   Samples were collected by snorkelers from the ten sampling sites in February–May 2012 and

123   February–March 2013 (Fig. 1). At each site, ten samples of macroalgal species (*Turbinaria* and/or

124   *Halimeda*) were collected with the surrounding seawater according to its availability from depths of

125   0.4–1 m. Macroalgae were collected carefully to minimize the loss of epiphytic dinoflagellates, and

126   placed in heavy duty Ziploc plastic bags, along with surrounding seawater. For sample processing, the

127   plastic bags containing the macroalgae were shaken vigorously for approximately one minute and the

128   suspension was sieved sequentially using 150 µm and 20 µm sieves. The macroalgae retained in the

129   150 µm sieve was blot dried with a paper towel and weighed. The material collected in the 20 µm

130   sieve was backwashed into a 50 ml falcon tube with 0.2 µm-filtered seawater, and brought to a volume

131   of 50 ml. 40 mL of this was transferred CytoOne tissue culture flask (USA Scientific Inc., Ocala,

132   Florida, USA) and used for cell isolations, and the remaining 10 mL was preserved in 4% formalin for

133   enumeration of the benthic dinoflagellate community.

134

135   *2.3 Environmental data*

136   Salinity, sea surface temperature (°C), and oxygen saturation (%) were measured using a CTD (Saiv

137   A/S, model SD204, Bergen, Norway) at six of the ten sampling sites (Fig. 1): Qita Al-Kirsh, Sh'ib

138   Nazar, Al Fahal, Abu Shosha, Um Al Balam, and Umm Al Kiethl. At each sampling site, the CTD was

139   positioned within 5 m of the area of the coral reef sampled, and measurements were collected just

140   below the sea surface (~ 20 cm). The average of 80 measurements collected from each sampling site

141   was calculated and used in subsequent analyses.

142   *2.4 Cell enumeration and statistical analyses*

143   The preserved samples were gently shaken and 0.5 ml-1 ml (depending on sample density) was

144   analyzed for benthic dinoflagellate abundance in a Sedgewick Rafter counting cell slide using a Leica

145   DM 2500 light microscope (Wetzler, Germany) at 100× magnification. The number of cells g^{-1} wet

146   weight macroalgae was calculated for each sample. Dinoflagellates in the genera *Gambierdiscus* and

147   *Ostreopsis* were identified by morphological features and enumerated. *Prorocentrum* and

148   *Amphidinium* cells were also observed in the samples, however, cell abundances of these genera were

149   not enumerated.

150   Statistical analyses were performed using SPSS 20.0 (SPSS, Chicago, Illinois, USA).

151   Kolgomorov-Smirnov testing was carried out on the cell abundances of both *Gambierdiscus* spp. and

152   *Ostreopsis* spp. at each sampling site to determine whether data were normally distributed. A non-

153   parametric Kruskall-Wallis test was used to determine whether there were significant differences in

154   *Gambierdiscus* spp. and *Ostreopsis* spp. mean cell abundances among the sampling sites. Mann-

155   Whitney U tests were carried out to see if there were significant differences between *Gambierdiscus*

156   spp. and *Ostreopsis* spp. median cell abundances at each sampling site.

157   *2.5 Culture establishment*

158   Unialgal cultures were established from the live samples by micropipetting and washing single

159   *Gambierdiscus* cells twice with sterile seawater to reduce contamination. Cultures were maintained in

160   modified K medium (Morton and Norris, 1990) at 25°C under cool white lights at a light intensity of

161   ~96 µmol m^{-2} s^{-1} with a 12h:12h light: dark cycle. All cultures examined in this study were isolated

162   from samples collected at the Sh'ib Nazar sampling site (Fig. 1).

163   *2.6 DNA sequencing of* Gambierdiscus *spp.*

164   DNA was extracted from ~1ml of dense culture using a MOBIO PowerSoil DNA isolation kit

165   (MOBIO, Carlsbad, California, USA) following the manufacturer's instructions, with a final elution

166   volume of 100 µl. The D8-D10 hypervariable region of the LSU rRNA was amplified using primers

167   FD8 and RB (Chinain *et al.*, 1999). PCR reactions (25 µl) contained ~2 ng template DNA, 1× PCR

168   Buffer (500 mM KCl and 100 mM Tris–HCl, pH 8.3), 2 mM MgCl$_2$, 0.8 mM dNTPs, 0.5 µM of each

169   primer, and 0.5 U of AmpliTaq DNA Polymerase (Applied Biosystems Inc., Foster City, California,

170   USA). Hot start PCR amplifications were performed using an Eppendorf Mastercycler Nexus PCR

171   system (Eppendorf, AG, Hamburg, Germany) as follows: 94°C for 4 min; then 35 cycles of 94°C for

172   30 s, 57°C for 1 min, 72°C for 2 min, and a final extension of 72°C for 10 min. Positive PCR products

173   were cloned into pGEM®-T Easy Vector using a pGEM cloning kit (Promega, Madison, WI, USA).

174   Clones were screened for inserts by PCR amplification with plasmid primers M13F and M13R and

175   positive clones from each PCR amplicon were selected for Sanger sequencing (Eurofins MWG

176   Operon, Ebersberg, Germany).

177   DNA sequences were manually edited and assembled using Geneious Pro 6.1.2 (Biomatters,

178   Auckland, NZ), and the consensus sequences were compared with those deposited in GenBank using

179   BLAST sequence similarity searches (National Center for Biotechnology Information).

180   *2.7* Gambierdiscus *spp. morphological identification*

181   Scanning electron microscopy (SEM) was used to examine thecal plate architecture and cell surface

182   morphology. For the SEM processing, approximately 10 ml of exponentially growing culture was

183   preserved with glutaraldehyde (2%); desalted with a ten step gradient from seawater (32‰) to

184   freshwater (90%, 80%, etc., to freshwater), followed by dehydration using a ten step gradient from

185 freshwater to 100% ethanol (10% ethanol, 20% ethanol, etc., to 100% ethanol), which was then

186 followed by a gradient of hexamethyldisilazane (HMDS). Samples were filter-mounted to a stub and

187 sputter coated with 1.5nm of gold-palladium (Denton Vacuum Desk II Sputter Unit, Moorestown, NJ,

188 USA). Measurements (length, width) of at least 25 cells observed were analyzed using MicroSuite

189 Five (Olympus, Japan). Parameters of cell depth and width, and size and shape of Apical Pore (Po), 1p,

190 2', and 4''' were measured. For consistency and ease of comparison of these results with the scientific

191 literature, *Gambierdiscus* were depicted by the plate tabulation nomenclature of Po, 3', 7'', 5''', 1p,

192 2'''' as described in the scientific literature (cingular and sulcus plates are not measured) (Faust, 1995;

193 Chinain *et al*., 1999; Litaker *et al*., 2009).

194

195 2.8 *Assessment of* Gambierdiscus *toxicity*

196 Three *Gambierdiscus* isolates were selected for assessment of toxin content; however, one culture

197 grew poorly and ultimately we were not able to achieve culture densities sufficient for analysis.

198 Between 2.0×10^6–1.0×10^7 cells from the other two batch cultures of *Gambierdiscus* were harvested

199 for toxicity detection in the early stationary phase. Ciguatoxins (CTXs) were extracted from

200 *Gambierdiscus* cell pellets according to the procedures described by Chinain *et al.* (2010) with some

201 modifications. Cell pellets were extracted in methanol under sonication for 30 min. After

202 centrifugation at 4000 rpm for 15 minutes, the supernatant was collected. The extraction was repeated

203 twice, and all the supernatant was combined. After the extract was evaporated, a solvent partition was

204 applied to the resulting residue three times using dichloromethane and 60% aqueous methanol. The

205 dichloromethane soluble fractions (DSFs), in which CTXs are recovered, were dried under vacuum

206 and stored at -20°C until tested for toxicity via mouse neuroblastoma assay (MNA).

207   Mouse neuroblastoma (Neuro- 2a cells) (ATCC, CCL131; ATCC, Manassas, VA) were cultured

208 in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Life Technologies, Carlsbad, CA)

209 that was supplemented with 10% fetal bovine serum (HyClone, Thermo Fisher Scientific, Waltham,

210 MA), 2 g/L Na_2CO_3, antibiotic solution (50 units/mL penicillin and 50 µg/mL streptomycin), and 2.5

211 µg/mL Fungizone® (Gibco Life Technologies, Carlsbad, CA) at 37°C in 5% CO_2. Cells were seeded at

212 a density of 2.5 x 10^5 cells/mL in 96-well plate. After 24 hr incubation, medium was renewed with

213 complete RPMI-1640 containing 0.1 mM ouabain and 0.01 mM veratridine. Cells were dosed with 10

214 uL per well extracts in three replicates. After 18 hr incubation, cell viability was measured by MTT [3-

215 (4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. Absorbance was measured using

216 a microplate reader (Spectra Max 340 PC, Molecular Devices Corporation, Sunnyvale, CA, USA) at

217 595 nm with a reference wavelength of 655 nm. The optical density acquired for each well was

218 normalized by the MTT blank.

219 Cells were dosed with 10 µL/well P-CTX-1 standards at seven concentrations ranging from 9.77

220 pg/mL to 78.1 pg/mL in five replicates. A standard curve of P-CTX-1 was plotted using non-linear

221 regression (R^2 < 0.990). Toxicities of *Gambierdiscus* spp. were determined based on the standard

222 curve with a limit of quantification (LOQ) ranged from 6.74 x 10^{-7} to 7.27 x 10^{-7} pg P-CTX-1 eq. cell^{-}

223 ^{1}. Quality control of the assay was performed by testing each MNA with P-CTX-1 standard of 39.1 pg

224 P-CTX-1 eq. The assays were conducted twice and the toxicity values are reported as mean P-CTX-1

225 eq between two assays. The intra-plate relative standard deviation ranged from 3.99% – 5.38%, and

226 inter-assay relative standard deviation was 8.40%.

227 In the scientific literature, ciguatoxicity of *Gambierdiscus* spp. is frequently expressed as P-CTX1

228 or P-CTX-3C equivalents depending on the ciguatoxin (CTX) used as a reference standard. Based on

229 the acute intraperitoneal injection (*i.p.*) LD$_{50}$ of P-CTXs in mice, the European Food Safety Authority

230 (EFSA) developed toxicity equivalency factors (TEFs) for individual P-CTXs, which can be applied to

231 express individual analogues identified with quantitative detection methods as P-CTX-1 equivalents

232 (EFSA Panel, 2010). The TEFs calculated for P-CTX-1 and P-CTX-3C are 1 and 0.2, respectively.

233 Therefore, the ciguatoxicity measured here (expressed as P-CTX-1 eq. cell^{-1}) was multiplied by its

234 corresponding TEF (i.e., 0.2) to enable general comparisons with other published toxicity

235 measurements expressed using P-CTX-3C equivalents.

236

237   **3. Results**

238   *3.1 Dinoflagellate abundance and distribution*

239   A total of 100 samples were collected from ten sites (Table 1 and Fig. 1) and analyzed for

240   abundance of *Gambierdiscus* and *Ostreopsis*. In general, cell abundances of *Gambierdiscus* spp. and

241   *Ostreopsis* spp. were low compared with Atlantic and Pacific observations (Fig. 2; e.g., Litaker *et al.*,

242   2010, Shears and Ross, 2009). Average cell abundances of *Gambierdiscus* spp. were higher in samples

243   collected from inshore reefs (Al Fahal, Abu Shosha, Um Al Balam, Um Al Kiethl, Mangrove Reef,

244   and Coast Guard Reef) compared with the offshore reefs (Qita Al-Kirsh, Sh'ib Nazar, Malathu Reef,

245   and Marmar Reef) (Table 2). Both the highest and lowest average cell abundances of *Ostreopsis* spp.

246   were observed in samples collected from offshore reefs (Sh'ib Nazar and Malathu reefs, respectively),

247   with highest average abundance of 143±45 observed at Sh'ib Nazar. Whereas *Ostreopsis* spp. cell

248   densities exhibited greater variability among offshore reefs (Fig. 2), abundances at the inshore reefs

249   were more uniform.

250   As some data were not normally distributed (Table 3), non-parametric tests were carried out to

251   determine whether there were significant differences in the average cell abundances of each genus

252   within and between the sampling reefs. Results from the Kruskall-Wallis tests indicated that at least

253   one sampling site was significantly different from the others in terms of median cell abundances (cells

254   g^{-1} of wet weight algae) of *Gambierdiscus* spp. and *Ostreopsis* spp. (Table 4). Mann Whitney U

255   comparison tests showed that median cell abundances of *Ostreopsis* spp. were significantly higher than

256   *Gambierdiscus* spp. at Abu Shosha, Um Al Balam, Um Al Kiethl, Sh'ib Nazar, Marmar Reef, Al

257   Fahal, Coast Guard Reef, and Mangrove Reef. (Table 5). There were no significant differences

258   between median cell abundances of *Gambierdiscus* spp. and *Ostreopsis* spp. at Qita Al-Kirsh and

259   Malathu (Table 4).

260   Analysis of the environmental data revealed a negative correlation between salinity and

261   average cell abundances for both genera (Fig. 3A, Fig. 4A), which was statistically significant for

262 *Gambierdiscus* spp. However, there were no statistically significant correlations between average sea

263 surface temperatures (°C) or average oxygen saturation (%) and *Gambierdiscus* spp. or *Ostreopsis* spp.

264 cell abundances (Fig. 3B and C; Fig. 4B and C).

265

266   *3.5 DNA sequencing*

267   DNA sequences were collected from nine *Gambierdiscus* isolates from the Red Sea (GenBank

268 Accession numbers: KY782637–KY782645). Consensus sequences were compared with those

269 deposited in GenBank using BLAST sequence similarity searches (National Centre for Biotechnology

270 Information, NCBI), which confirmed that all isolates sequenced from the Red Sea were closely

271 related to *G. belizeanus*. Genetic distance values between sequences from the Red Sea isolates

272 compared with conspecific sequences from GenBank (Caribbean isolates) ranged from 0.005-0.015.

273 Distance values among sequences from the Red Sea isolates ranged from 0.003-0.012, with the

274 exception of isolate RS2-B6. All clones sequenced from this isolate were likely pseudogenes, and

275 contained a 116 bp deletion at positions 493-609 (compared with *G. belizeanus*, EU770672). After

276 excluding this section, genetic distance values between RS2-B6 and other Red Sea isolates ranged

277 from 0.021-0.01, and distance values between RS2-B6 and conspecific sequences from GenBank were

278 0.012-0.019.

279 *3.4* Gambierdiscus *morphology*

280 The morphology of six isolates of *Gambierdiscus belizeanus* was examined using LM and SEM to

281 provide a characterization of this species in the Red Sea (Fig. 5, 6). All isolates featured an antero-

282 posteriorly compressed shape covered with numerous evenly spaced pores. The thecal plates were

283 deeply areolated and could be distinguished under light microscopy (Fig. 5). The epitheca consisted of

284 11 plates, was oriented ventrally, and contained the typical fishhook-shaped apical pore (Fig. 6A-C).

285 The cingulum consisted of six narrow plates and displayed a curved end located at the edge of the

286 sulcal opening. The sulcus was broad and situated between the epithecal and hypothecal plates. The

287   hypotheca consisted of eight plates, with the 1p plate being long and narrow (Fig. 6E-F). All characters

288   of these six isolates were consistent with the description of *G. belizeanus* (Faust, 1995).

289

290   *3.6 Gambierdiscus toxicity*

291   Two isolates identified as *G. belizeanus* were analyzed for CTX-like toxicity. Both strains produced

292   toxins; however, the toxin contents or cell quotas were dissimilar. The toxin content of *G. belizeanus*

293   RS2-B6 was $6.50 \times 10^{-5} \pm 1.14 \times 10^{-5}$ pg P-CTX-1 eq. cell^{-1}, and the toxin content of RS3-B8 was 1.02

294   $\times 10^{-5} \pm 2.71 \times 10^{-6}$ pg P-CTX-1 eq. cell^{-1}. The limit of quantification ranged from 6.74×10^{-7} to 7.27

295   $\times 10^{-7}$ pg P-CTX-1 eq. cell^{-1}. To compare toxin contents expressed as P-CTX-1 eq. cell^{-1} with other

296   published toxicity measurements expressed using P-CTX-3C equivalents, the average values were

297   multiplied by the appropriate TEF (i.e., 0.2; EFSA Panel, 2010). Toxin contents of RS2-B6 and RS3-

298   B8 expressed as P-CTX-3C were 1.3×10^{-5} P-CTX-3C eq. cell^{-1} and 2.04×10^{-6} P-CTX-3C eq. cell^{-1}.

299

300   **4. Discussion**

301   This study confirms the presence of epiphytic benthic dinoflagellates associated with ciguatera fish

302   poisoning (CFP) in the central Red Sea. Morphological analyses using light microscopy (LM)

303   identified the genera *Gambierdiscus* and *Ostreopsis* from collected samples, and further examination

304   of unialgal cultures using scanning electron microscopy (SEM) and DNA sequencing identified

305   multiple isolates of the species *G. belizeanus*. The two isolates of *G. belizeanus* that were analyzed

306   were both toxin-producers.

307   *4.1 Dinoflagellate abundance and distribution.*

308   Previous field investigations in regions where ciguatera-implicated dinoflagellates and CFP are

309   prevalent have reported maximum cell densities of *Gambierdiscus* spp. ranging from an average of

310   less than 100 to over 100,000 *Gambierdiscus* cells g^{-1} algae in the Pacific and Caribbean regions, with

311   a slightly higher frequency of high density samples in the Atlantic (reviewed by Litaker *et al*., 2010).

312   Several studies have identified finely branched or filamentous macroalgae as harboring particularly

313   high *Gambierdiscus* abundances (e.g., Parsons and Preskitt, 2007), while others reported high cell

314   concentrations on calcified algae (such as the taxa collected in this study; e.g., Bomber *et al.,* 1989;

315   Yasumoto *et al.,* 1979), and even on gas-filled, globos taxa (e.g., Heil *et al*., 1998). Efforts to identify

316   particular "preferred" algal substrates within reef habitats have been complicated by methodological

317   approaches associated with comparing cell counts normalized to the weight of the macroalgal host

318   among algal taxa with different surface area to mass ratios.

319   In contrast with abundance estimates reported from reef systems in other parts of the world, the

320   maximum number of *Gambierdiscus* cells encountered in this study was comparatively low (< 40 cells

321   g^{-1} wet weight algae). It is also evident from the present study that *Ostreopsis* spp. were more abundant

322   than *Gambierdiscus* spp. (Fig. 4) during the sampling period, which is similar to reports from other

323   reef systems (e.g., Tindall and Morton, 1998; Parsons and Preskitt, 2007; Richlen and Lobel, 2011).

324   The maximum cell abundance of *Ostreopsis* spp. was an order of magnitude higher than

325   *Gambierdiscus*, but still considerably lower than cell abundances reported elsewhere, which can reach

326   extraordinarily high concentrations under certain conditions (e.g., $> 1.4 \times 10^6$ cells g^{-1}; Shears and

327   Ross, 2009). If these low cell densities are representative of *Gambierdiscus* communities elsewhere in

328   the Red Sea, this may partly explain the lack of reports of CFP in this region, or negative impacts

329   associated with benthic blooms of *Ostreopsis*. A visit to any local fish market on the Saudi Arabian

330   Red Sea coast clearly indicates that there are no local taxonomic restrictions on what species of fish

331   are eaten, as very large individuals of grouper, snapper, and barracuda species are sold and consumed

332   indiscriminately. However, this study was conducted in a geographically restricted region of the Red

333   Sea and over a brief period from February-May 2012 and from February-March 2013. Therefore, the

334   results do not capture any potential seasonal variability in the population dynamics, which has been

335   observed in both the Pacific and the Caribbean. In Tahiti, Chinain *et al.* (1999) documented seasonal

336   cycles of *Gambierdiscus* spp. cell densities, with cell abundances reaching their maximum at the end

337   of the hot season. Similarly, monthly sampling carried out in Hawaii over a period of 3.5 years

338   documented seasonal changes in abundance, with highest cell densities observed during the summer

339   months (Parsons *et al.*, 2010). These results were consistent with previous observations in Australia

340   (Gillespie *et al.*, 1985) and in the Florida Keys (Bomber *et al.*, 1988). Therefore, it is possible that

341   there may also be seasonal cycles in *Gambierdiscus* and *Ostreopsis* cell abundances in the central Red

342   Sea, where minimum temperatures of ~25°C occur between January and March and maximum

343   temperatures exceeding 32°C are common in late August (Davis *et al.*, 2011). This could easily be

344   confirmed through long-term surveys.

345   Natural and anthropogenic disturbances may contribute to the risk of CFP by increasing the

346   amount of benthic substrate available to harbor epiphytic dinoflagellates (Hallegraeff, 1993; Cruz-

347   Rivera and Villareal, 2006). In 2010 a major bleaching event in the study region impacted inshore

348   reefs more than offshore reefs. Inshore reefs near Thuwal experienced 50-100% bleaching of corals at

349   5 m depth while offshore reefs in the region experienced 5-30% bleaching at the same depth (Furby *et*

350   *al.*, 2013). Furthermore, corals on offshore reefs appeared to recover from bleaching, whereas the

351   corals on inshore reefs appeared to suffer very high mortality rates (Furby *et al.*, 2013). Therefore, the

352   availability of newly created substrate (dead coral surfaces) may have favored the proliferation of

353   benthic dinoflagellates on the inshore reefs (Kohler and Kohler, 1992); however, baseline or time-

354   series data to further support this hypothesis are unavailable. Although spatial data regarding fishing

355   effort are not available for this region, fishing pressure may be higher on reefs closer to shore, as local

356   fishermen use small, open boats with limited range (Jin *et al.*, 2012). These two known local

357   disturbances could have increased substrate for the growth of macroalgal hosts for epiphytic

358   dinoflagellates, potentially increasing the prevalence of ciguatoxic fishes in inshore reefs. The present

359   study was unable to investigate potential CFP reports in local hospitals and clinics, but future work in

360   this area may be helpful to determine if cases are simply underreported or if they are truly rare or non-

361   existent in the region.

362   *4.2* Gambierdiscus *spp. physiology and environmental conditions in the Red Sea.*

363   The Red Sea is an oligotrophic, narrow, shallow basin (reaching a maximum depth of about 2300 m)

364   (Raitsos *et al*., 2013). It is one of the warmest and most saline seas in the world, with seasonal

365   temperatures ranging from 25ºC to 32ºC (Sofianos and Johns, 2007; Raitsos *et al.*, 2013), and salinities

366   ranging from ~36 to well over 40 at the northern end (Edwards, 1987). Based on what is currently

367   known regarding their ecology, species of *Gambierdiscus* are likely to be found in shallow water

368   habitats (< 100 m) with abundant macrophytes, algal turfs, or biofilms to which cells can attach;

369   annual temperatures ranging between 21–31°C; high, stable salinities; at light levels <10% of surface

370   irradiance; and sufficient nutrient inputs. There are, however, many biological differences among

371   various *Gambierdiscus* species, which potentially lead to differences in their ecological preferences

372   (thus influencing their geographic distributions). At the sites sampled in this study, both genera were

373   negatively correlated with salinity (Figs. 3A and 4A); however, laboratory experiments indicate that

374   *Gambierdiscus* has a wide range of tolerance for salinity (Bomber *et al*., 1988; Tindall and Morton,

375   1998; Kibler *et al*., 2012; Xu *et al*., 2016) and thus may be able to persist in the extreme salinity levels

376   observed in the Red Sea. Physiological studies by Kibler *et al.* (2012) indicated that salinities less than

377   36 supported positive growth of most Caribbean *Gambierdiscus* species, and concluded that salinities

378   surpassing this threshold may impose a metabolic cost to *Gambierdiscus* cells. However, in an

379   examination of multiple *G. belizeanus* isolates from the Caribbean Sea, maximum growth was

380   observed across a range of salinities from 30.3-36.6 and the optimum growth range for these isolates

381   included salinities >40, indicating robust growth at relatively high salinity levels (Xu *et al*., 2016).

382   Based on these data, other environmental factors (e.g., temperatures or light availability) associated

383   with shallow reef habitats may account for the observed difference in *Gambierdiscus* spp. cell

384   abundance between inshore and offshore reefs.

385   Laboratory and field studies examining thermal tolerances of multiple *Gambierdiscus* species

386   suggests a preference for temperatures ranging from ~24–34ºC, with optimum growth generally

387   observed from ~25–31°C (e.g., Litaker *et al*., 2010; Kibler *et al*., 2012; Xu *et al*., 2016). Specifically

388   for *G. belizeanus*, Xu *et al*. (2016) reported that temperatures ranging from 23.1–32.3ºC supported

389 optimal growth of four isolates, while Kibler *et al*. (2012) reported a similar, but slightly narrower

390 optimum growth range of 24.7–30.4ºC for a single *G. belizeanus* isolate. Slight differences in

391 temperatures markedly affect growth potentials of all *Gambierdiscus* species, with declines observed

392 at temperatures exceeding 30-31ºC (Kibler *et al*., 2012; Xu *et al*., 2016). Many reefs in the Red Sea

393 experience maximum temperatures above 31ºC in the summer months with a minimum temperature of

394 around 26ºC during the winter months (Sofianos and Johns, 2003). The presence of *G. belizeanus* in

395 the central Red Sea indicate that populations in this region may have some physiological plasticity

396 exceeding the aforementioned limits, particularly with respect to temperature, or possibly that some

397 level of local adaptation is occurring. A comparison of conspecific isolates from this region would help

398 establish if physiological tolerances affecting growth are similar, or if Red Sea populations are adapted

399 to local environmental conditions.

400 The inhospitable conditions that are seasonally present may also partly explain the low cell

401 densities of *Gambierdiscus* and *Ostreopsis* compared with regions such as the Caribbean and Pacific.

402 Further work investigating the specific mechanisms involved in these species' abilities to cope with

403 higher salinities and temperatures could provide insight into their inherent capacity to deal with future

404 global climate scenarios.

405 *4.6 Analysis of* Gambierdiscus belizeanus *toxin content*

406 A survey by Chinain *et al*. (2010) of the toxicity of various *Gambierdiscus* species in Tahiti found the

407 highest toxicity in *G. polynesiensis* (0.017–4.4 pg P-CTX-3C eq. cell^{-1}; n=4), and the lowest in *G.*

408 *pacificus* (not detected; n=3), *G. toxicus* (not detected–0.028 pg P-CTX-3C eq. cell^{-1}; n=5), and *G.*

409 *australes* (not detected–0.03 P-CTX-3C eq. cell^{-1}; n=6). The toxicity of the *G. belizeanus* strain from

410 Tahiti was 0.0246 P-CTX-3C eq. cell^{-1}. In a separate study conducted in the Cook Islands, Rhodes *et*

411 *al.* (2010) reported that the toxin content of a strain of *G. australes* was 0.04 pg CTX-1 eq cell^{-1}.

412 Compared to the aforementioned strains of *Gambierdiscus* from these areas, the toxin content of Red

413 Sea isolates is considerably lower. Isolate RS2-B6 was toxic in the MNA test at 6.50×10^{-5} pg P-CTX-

414 1 eq. cell^{-1} (1.3×10^{-5} P-CTX-3C eq. cell^{-1}) and the toxin content of RS3-B8 was 1.02×10^{-5} pg P-

415 CTX-1 eq. cell^{-1} (2.04×10^{-6} P-CTX-3C eq. cell^{-1}). Thus, the results from this study indicate that

416 although Red Sea dinoflagellates produce CTX, the low *Gambierdiscus* densities observed, along with

417 their comparatively low toxin contents may explain why CFP is largely unknown and unreported in

418 this region. The data presented here and by Saburova *et al.* (2013) have shown that toxin-producing

419 species are present in the region, and at certain locations could potentially contribute to toxicity in reef

420 fish. However, given the limited temporal and spatial scale of this investigation, additional studies are

421 needed to provide a fuller characterization of the diversity and toxicity of *Gambierdiscus* species in the

422 region, and whether ciguatoxins are entering the food web.

423 **5. Conclusions**

424 The current difficulties in predicting, detecting, and treating CFP indicate that this disease will

425 continue to have significant socioeconomic impacts, especially in developing countries (Lewis., 2001).

426 Potential impacts will be greatest in regions where subsistence fishing is prevalent. Despite recent

427 advances in the characterization of the taxonomy and geographic distribution of *Gambierdiscus* spp.,

428 very little is known about the distribution of this species and other toxigenic benthic dinoflagellates or

429 the prevalence of CFP in the Red Sea and Arabian Gulf region. This study is one of the first to

430 describe the composition and distribution of these communities in the Red Sea. However, assessing the

431 risk of CFP in the region is impossible without additional data regarding the distribution and

432 composition of the benthic dinoflagellate community over larger areas and time frames, the taxonomy

433 of *Gambierdiscus* spp. throughout the region, or the chemical structure of the toxins they produce. This

434 study is a step in that direction, but further studies are needed, particularly those that would document

435 toxin levels in fish from Red Sea reefs, as well as the incidence of CFP intoxication from the

436 surrounding countries.

437

438 **Acknowledgements**

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608 **Table 1**. Summary of the macroalgae samples collected at ten sampling sites in the central Red Sea, Saudi Arabia, including sampling stations,
609 collection date, collection depth, number of macroalgae samples collected, macroalgae species collected, and CTD data from each sampling site.
610 ND=no data.

Sampling station	Collection date	No.macroalgae samples collected	Macroalgal species collected	Sampling depth (m)	Seawater Temperature (°C)	Salinity	Oxygen Saturation (%)
Qita Al - Kirsh	26/02/2012	2	*Turbinaria decurrens* and *Halimeda sp*	0.5	29.3	38.4	25.3
	15/05/2012	5	*Turbinaria decurrens*	0.6			
	24/02/2013	3	*Turbinaria decurrens* and *Halimeda sp.*	0.5			
Sh'ib Nazar	27/02/2012	5	*Turbinaria decurrens*	0.4	30.1	28.9	8.6
	15/05/2012	5	*Turbinaria decurrens*	0.4			
Malathu Reef	17/04/2012	5	*Turbinaria decurrens*	0.5	ND	ND	ND
	05/03/2013	5	*Turbinaria decurrens*	0.7			
Al Fahal	15/05/2012	5	*Turbinaria decurrens* and *Halimeda sp.*	0.8	30.4	36.0	1.73
	24/02/2013	5	*Turbinaria decurrens* and *Halimeda sp.*	0.6			
Abu Shosha	16/05/2012	5	*Turbinaria decurrens* and *Halimeda sp.*	0.5	29.6	38.3	12.5
	25/02/2013	5	*Turbinaria decurrens* and *Halimeda sp.*	0.5			
Um Al Balam	16/05/2012	5	*Turbinaria decurrens* and *Halimeda sp.*	0.6	29.3	36.1	32.1
	27/03/2013	5	*Turbinaria decurrens* and *Halimeda sp.*	0.4			
Um Al Kiethl	16/05/2012	5	*Turbinaria decurrens* and *Halimeda sp.*	0.5	29.5	28.9	23.1
	25/02/2013	5	*Turbinaria decurrens* and *Halimeda sp.*	0.5			
Marmar Reef	05/03/2013	10	*Turbinaria decurrens*	0.8	ND	ND	ND
Coast Guard Reef	06/03/2013	10	*Turbinaria decurrens* and *Halimeda sp.*	0.5	ND	ND	ND
Mangrove Reef	23/03/2013	10	*Turbinaria decurrens*	0.4	ND	ND	ND
Total		100					

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614   **Table 2.** Kolgomorov-Smirnov test to determine whether *Gambierdiscus* spp. and *Ostreopsis* spp. cell

615   abundances were normally distributed.

Sampling reef	p value, *Gambierdiscus* spp.	Normally distributed?	p value, *Ostreopsis* spp.	Normally distributed?
Qita Al - Kirsh	p = 0.073	Yes	p = 0.032	No
Sh'ib Nazar	p = 0.200	Yes	p = 0.061	Yes
Malathu	p = 0.200	Yes	p = 0.200	Yes
Marmar	p = 0.009	No	p = 0.200	Yes
Al Fahal	p = 0.200	Yes	p = 0.011	No
Abu Shosha	p = 0.001	No	p = 0.000	No
Um Al Balam	p = 0.200	Yes	p = 0.000	No
Um Al Kiethl	p = 0.050	Yes	p = 0.082	Yes
Coast Guard Reef	p = 0.200	Yes	p = 0.200	Yes
Mangrove Reef	p = 0.001	No	p = 0.021	No

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619 **Table 3.** Kruskall-Wallis test for significant differences in the median cell abundances of

620 *Gambierdiscus* spp. and *Ostreopsis* spp. among ten sampling sites in the Saudi Arabian Red Sea. The

621 numbers in brackets represent the confidence intervals used for the analyses.

Species	Kruskall-Wallis test	p value
Gambierdiscus spp.	$X^2_{(9)} = 37.314$	$p < 0.001$
Ostreopsis spp.	$X^2_{(9)} = 63.281$	$p < 0.001$

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Table 4. Comparisons between *Gambierdiscus* spp. median cell abundance and *Ostreopsis* spp. median cell abundance at ten sampling sites in the central Saudi Arabian Red Sea. The numbers in brackets represent the number of sampling sites used for the analyses. (NS = not significant, HSD = highly significant difference, SD = significant difference.)

Sampling site	Mann Whitney U test	Significance
Qita Al - Kirsh	$U_{(10)} = 40.5$, $P > 0.05$	NS
Sh'ib Nazar	$U_{(10)} = 2.0$, $P < 0.001$	HSD
Malathu Reef	$U_{(10)} = 43.5$, $P > 0.05$	NS
Marmar Reef	$U_{(10)} = 0.0$, $P < 0.001$	HSD
Al Fahal	$U_{(10)} = 5.5$, $P = 0.001$	HSD
Abu Shosha	$U_{(10)} = 17.0$, $P < 0.05$	SD
Um Al Balam	$U_{(10)} = 13.0$, $P < 0.05$	SD
Um Al Kiethl	$U_{(10)} = 16.0$, $P < 0.05$	SD
Coast Guard Reef	$U_{(10)} = 0.0$, $P < 0.001$	HSD
Mangrove Reef	$U_{(10)} = 7.5$, $P = 0.001$	HSD

659   **Table 5.** Mann Whitney U comparisons of *Gambierdiscus* sp. and *Ostreopsis* spp. median cell

660   abundances from samples collected on inshore and offshore reefs in the central Saudi Arabian Red

661   Sea. The numbers in brackets represent the number of samples used for analyses.

662

Species	Mann Whitney U test	p value
Gambierdiscus spp.	$U_{(100)} = 628.5$	$p < 0.001$
Ostreopsis spp.	$U_{(100)} = 696.0$	$p < 0.001$

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666

667 **Figure Captions**

668 **Figure 1.** Sampling sites used in this study included six coral reefs off the coast of Thuwal and four

669 coral reefs off the coast of Al-Lith, Saudi Arabia. Macroalgae collected at these reefs were examined for

670 the presence of ciguatera-associated benthic dinoflagellates.

671 **Figure 2.** Average cell abundance of *Gambierdiscus* and *Ostreopsis* spp. from ten sampling sites in the

672 central Saudi Arabian Red Sea, as determined by visual counts and standardized to number of cells^{-1} g

673 of wet weight of host macroalgae. Bars: mean ± 1 SE.

674 **Figure 3.** Relationships between average *Gambierdiscus* spp. cell abundance and A) average salinity

675 (p= 0.02), B) sea surface temperature (°C) (p= 0.25) and C) oxygen saturation (%) (p= 0.29) at six

676 sampling sites (Qita Al – Kirsh, Sh'ib Nazar, Al Fahal, Abu Shosha, Um Al Balam, and Um Al Kihel)

677 in the central Saudi Arabian Red Sea.

678 **Figure 4.** Relationships between average *Ostreopsis* spp. cell abundance and A) average salinity (p=

679 0.11), B) sea surface temperature (°C) (p= 0.29) and C) oxygen saturation (%) (p= 0.28) at six sampling

680 sites (Qita Al – Kirsh , Sh'ib Nazar, Al Fahal, Abu Shosha, Um Al Balam, and Um Al Kihel) in the

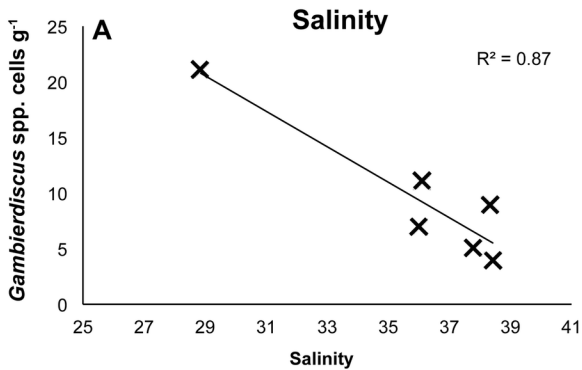681 central Saudi Arabian Red Sea.

682 **Figure 5.** Light microscope images of *Gambierdiscus belizeanus* from live samples collected

683 off the coral reefs in the central Saudi Arabian Red Sea. Scale bars are 20µm.
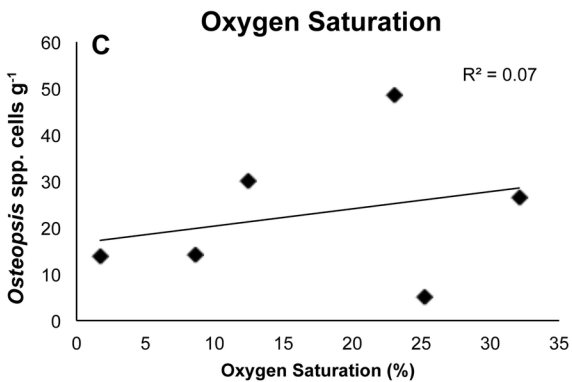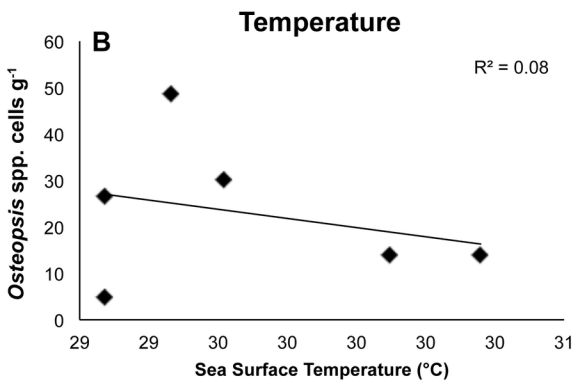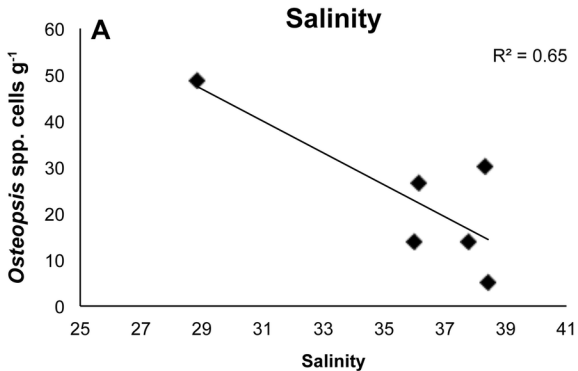
684 **Figure 6.** Scanning electron micrographs of *Gambierdiscus belizeanus* sampled from the central Saudi

685 Arabian Red Sea. A) Strain RS2-B6: Multiple cells, with mucous evident; B) Strain RS2-B8, epitheca;

686 C) Strain RS2-B6, Ventral view; D) Strain RS2-B2; epitheca; E) View of hypotheca; F) RS3-B8: View

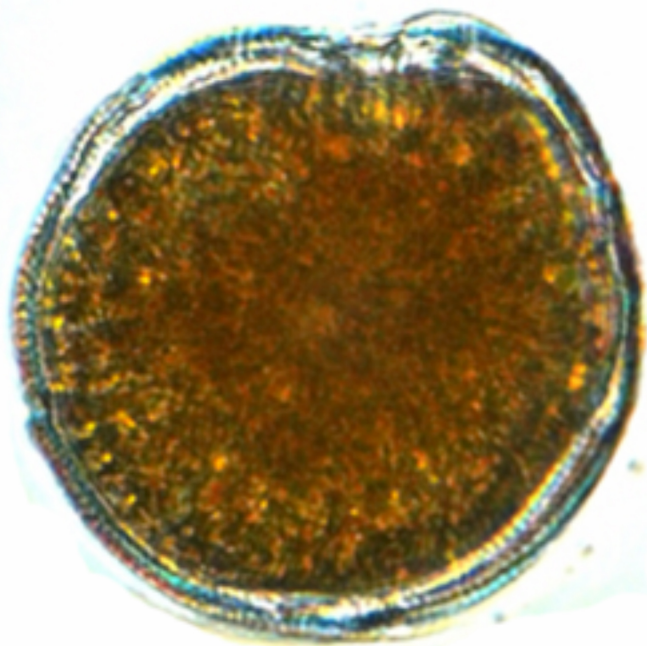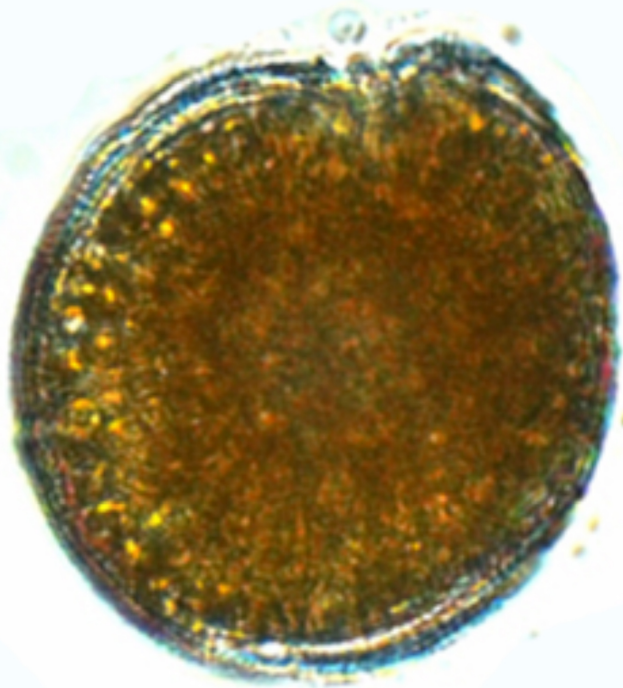687 of hypotheca. Scale bars are 10µm (except for A where the scale bar is 20µm).

688

Saudi Arabia, Egypt, Sudan, Eritrea

Thuwal, Jeddah, Al Lith

Qita Al Kirsh, Sh'ib Nazaar, Abu Shosha, Al Fahal, Thuwal, Um Al Balam, Umm Al Kidai

Al Lith, Malathu Reef

Gambierdiscus spp.

Ostreopsis spp.

A Salinity

$R^2 = 0.87$

Gambierdiscus spp. cells g^{-1} (y-axis)
Salinity (x-axis)

B Temperature

$R^2 = 0.10$

Gambierdiscus spp. cells g^{-1} (y-axis)
Sea Surface Temperature (°C) (x-axis)

C Oxygen Saturation

$R^2 = 0.12$

Gambierdiscus spp. cells g^{-1} (y-axis)
Oxygen Saturation (%) (x-axis)

A Salinity — $R^2 = 0.65$
B Temperature — $R^2 = 0.08$
C Oxygen Saturation — $R^2 = 0.07$

A

B

C

A.

C

D

2"

1'

6"

Po

3'

2'

5"

3"

4"