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Isolation and growth in culture of three *Gambierdiscus* species from central-southern Cuba

GLEISY PÉREZ-AVILLEIRA[1], ÁNGEL R. MOREIRA-GONZÁLEZ[1], GABRIEL L. ROJAS-ABRAHANTES[1], DONAIDA CHAMERO-LAGO[1], MARIE-YASMINE DECHRAOUI BOTTEIN[2,3], CARLOS ALONSO-HERNÁNDEZ[1,2] & LISBET DÍAZ-ASENCIO[1*]

[1] *Centro de Estudios Ambientales de Cienfuegos, Carretera Castillo de Jagua Km 1½, Ciudad Nuclear, Cienfuegos, CP. 59350, Cuba.*
[2] *Environment Laboratories, International Atomic Energy Agency, 4 Quai Antoine 1er, MC 98000 Monaco.*
[3] *Ecotoxicologie et Développement Durable Expertise (ECODD), 1 passage des pignes, 06560 Valbonne, France*

*Corresponding author: lisbet@ceac.cu

Abstract. *Gambierdiscus* genus is the main causative agent of ciguatera poisoning, a foodborne illness caused by the ingestion of fish contaminated with ciguatoxins. Data acquisition on *Gambierdiscus* occurrence, identification, enumeration and toxicity are fundamental in ciguatera research and risk management. It strongly relies on the capacity to isolate and culture these species in a laboratory setting. In this study, the performance of *Gambierdiscus* cell isolation and culture process was assessed through evaluation of success rate from six water samples collected in four sites in the central-southern region of Cuba. Out of 352 cells isolated, 28 strains were successfully maintained in culture. The strains belonged to three *Gambierdiscus* species, *G. belizeanus*, *G. carolinianus* and *G. caribaeus*. The success rate of isolation ranged from zero to 29.7 % and averaged 8 % in the six seawater samples analyzed. Growth rate values ranged from 0.14 days^{-1} for both *G. belizeanus* and *G. carolinianus* to 0.17 days^{-1} for *G. caribaeus*. Significant differences were recorded between species for the maximum cell density measured at stationary phase. This study points at the difficulties in culturing wild strains of *Gambierdiscus* and contributes to basic studies on the growth of *Gambierdiscus* species needed for the ongoing toxicological researches in the central-southern coast of Cuba.

Key words: benthic dinoflagellates, ciguatera, *Gambierdiscus* genus, growth rate, microalgal isolation.

Resumen: Aislamiento y crecimiento en cultivo de tres especies de *Gambierdiscus* de la región centro-sur de Cuba. El género *Gambierdiscus* es el principal agente causal de la ciguatera, intoxicación debida al consumo de peces contaminados con ciguatoxinas. La recopilación de datos y la generación de información sobre la ocurrencia, identificación, enumeración y evaluación de toxicidad de *Gambierdiscus* son fundamentales para el manejo de esta enfermedad, lo cual depende en gran medida en la capacidad de aislar y cultivar estas especies en condiciones de laboratorio. En este estudio se investigó el proceso de aislamiento y cultivo de *Gambierdiscus* a través de la evaluación de la tasa de obtención de cultivos a partir de seis muestras colectadas en cuatro sitios de la región centro-sur de Cuba. De las 352 células aisladas 28 cepas fueron mantenidas con éxito en cultivo. Las cepas aisladas correspondieron a las especies *G. belizeanus*, *G. carolinianus* y *G. caribaeus*. La tasa de crecimiento varió entre 0.14 días^{-1} para *G. belizeanus* y *G. carolinianus*, y 0.17 días^{-1} para *G. caribaeus*. Se registraron diferencias significativas entre especies para la máxima densidad celular obtenida en la fase estacionaria. Este estudio enfatiza en las dificultades de cultivar cepas de *Gambierdiscus* y

contribuye con los estudios básicos en curso sobre el crecimiento de especies de *Gambierdiscus*, necesarios para las investigaciones toxicológicas en la región centro sur de Cuba.

Palabras clave: aislamiento de microalgas, ciguatera, dinoflagelados bentónicos, género *Gambierdiscus*, tasa de crecimiento.

Introduction

Ciguatera poisoning is an endemic disease from tropical and subtropical regions, mostly occurring after consumption of reef fish contaminated with ciguatoxins. Its main causative agents are species of benthic dinoflagellates belonging to the genera *Gambierdiscus* and *Fukuyoa*. These organisms grow on a variety of benthic substrates, such as the surface of macroalgae, algal turfs, rocks or soft sediments substrates, in tropical and sub-tropical waters. To date, 19 species of the genus *Gambierdiscus* have been described worldwide, based mostly on morphological and molecular studies. So far, eight species of *Gambierdiscus*/*Fukuyoa* have been recorded in the Caribbean region: *Gambierdiscus belizeanus*, *Gambierdiscus caribaeus*, *Gambierdiscus carolinianus*, *Gambierdiscus carpenteri*, *Gambierdiscus excentricus*, *Gambierdiscus silvae*, *Fukuyoa ruetzleri*, and *Gambierdiscus* ribotype2, not yet described (Faust 1995, Litaker *et al*. 2009, Fraga & Rodríguez 2014).

The potent ciguatoxins (CTX) these dinoflagellates produce are transferred through the food chain, from small herbivorous fish or invertebrates to bigger fish or mammals that feed on them (Lehane & Lewis 2000, Dechraoui Bottein *et al.* 2011). Contaminated fish and shellfish are not affected in appearance, color, texture and taste what make impossible the detection of the toxins by organoleptic properties. Ciguatoxins are extremely stable, so that heat, freezing, dehydration, salting, or other processes do not affect their toxicity (Lehane & Lewis 2000). The acute signs and symptoms in humans commonly include gastrointestinal distress such as abdominal cramps, diarrhea, nausea, and vomiting followed by neurological symptoms that may relapse for weeks or months. Additional chronic symptoms include neurological and neuropsychiatric disorders that may persists for months or years (Chinain *et al.* 2019). Each year up to 25,000-50,000 cases of ciguatera poisoning are reported globally; however, the true number of cases is difficult to ascertain due to underreporting or misdiagnosis (Friedman *et al*. 2017).

In addition to epidemiological and CTX-fish contamination surveillance, integrated ciguatera risk management plan should involve the surveillance of the causative organisms *Gambierdiscus* and *Fukuyoa* (IOC/IPHAB 2015). Methods have been developed to assess the abundance of *Gambierdiscus* and *Fukuyoa* in the environment, involving natural and artificial substrate collection (Tester *et al.* 2014). From such samples, *Gambierdiscus* and *Fukuyoa* cells can be enumerated. However, the establishment and maintenance of clonal cultures from individual cells is critical for a proper species identification and toxicity assessment.

Culture of the dinoflagellates *Gambierdiscus* and *Fukuyoa* is one of the most challenging and arduous tasks when dealing with marine phytoplankton in laboratory (Assunção *et al*. 2019). The species of these two genera are characterized by slow growth and some of them have narrow tolerance ranges of salinity, temperature and irradiance. Several studies have reported maximum and optimal culture conditions for *Gambierdiscus* species (temperature, salinity, and irradiance) (Tester *et al*. 2010, Kibler *et al*. 2012, Xu *et al*. 2016). Toxin production in the different growth phases has been also studied for some species (Chinain *et al*. 2010).

However, at present there are no studies that address the difficulties in culturing benthic dinoflagellates, especially of *Gambierdiscus*/*Fukuyoa* species and success rates have seldom been published (Lozano-Duque *et al.* 2018). This work aimed at assessing the success rate of cell isolation, growth and maintenance of *Gambierdiscus* species isolated from central-southern region of Cuba. This information is important to characterize the species-specific distribution and toxicity of *Gambierdiscus* in order to better design a surveillance system to assess ciguatera threat in Cuba and the Caribbean region.

Materials and methods

Sampling sites and collection: Macroalgae samples were collected in four different sites along the central-southern region of Cuba during three sampling campaigns carried out in 2016-2018. Three sites were located in the coast of Cienfuegos province: Mangles Altos (22°03′19″N 80°30′16″W), Delfinario (22°02′16″N 80°25′45″W) and Guajimico

(21°55′38″N 80°18′36″W); and one site was located in a reef off the coast near Cayo Guano del Este (21°40′13″N 81°02′18″W) (Díaz-Asencio *et al*. 2019a, Díaz-Asencio *et al*. 2019b) (Fig. 1). Fronds of different macroalgae species were gently collected to prevent the detachment of dinoflagellates, and placed into 500 mL glass bottles with surrounding seawater. Approximately 1/3 of the seawater content in each glass bottle was removed to facilitate a vigorous hand-shaking (1-2 minutes) for the detachment of dinoflagellates associated with macroalgae. Afterwards, the seawater was filtered through a 200 µm mesh to remove gross materials (debris and macroalgae). When isolation right after sampling was not possible samples were properly kept in a culture chamber (EGCS 301, EQUITEC) at 26 °C.

Cell isolation and culture conditions: Single *Gambierdiscus*/*Fukuyoa* cells were isolated from the macroalgae material using a glass Pasteur pipette under an inverted microscope (Andersen 2005). The capillary pipette was drawn to a narrow filament, verifying first that the tip had a blunt shape to avoid physical damage to the isolated cells. Approximately 10-20 cells were transferred to a small Petri dish containing 1 mL of original seawater filtered with a 0.2 µm polycarbonate filter. Subsequently, the cells were re-isolated in five sequential drops of sterile seawater to remove any other different microalgal cell before being transferred to individual wells of a 96-multiwell plate containing 150 µL of sterile seawater.

The plate was sealed with parafilm in order to avoid evaporation of seawater and placed in a culture chamber (EGCS 301, EQUITEC) at 26 °C, 50-100 µmol photons m^{-2} s^{-1} of fluorescent white light with a day:night photoperiod of 12:12 hours as per Litaker *et al.* (2009) recommendations. After several cell divisions assessed by microscopy, the new cells were transferred to a 24-multiwell plate containing sterile seawater in which drops of 50 % f10k culture medium (Holmes *et al.* 1991) with salinity adjusted to 32 were added progressively every week. As the density of the culture increased above 100 cells $well^{-1}$ approximately, the cells were subsequently transferred to 50 mL glass Erlenmeyer flasks containing 15 mL of sterile seawater and 5 mL of 100 % f10k medium under the same environmental conditions. For culture maintenance, cells were transferred every three weeks to sterile 50 mL glass Erlenmeyer flasks containing fresh f10k medium. As cultures were transferred to new containers, the proportion of medium was gradually increased in the next three transfer steps adding 10, 15 mL and 20 mL of 100 % f10k until the cells were growing in 100 % f10K medium. The success rate of the cell isolation process was calculated as the ratio between the number of successful cultures and the

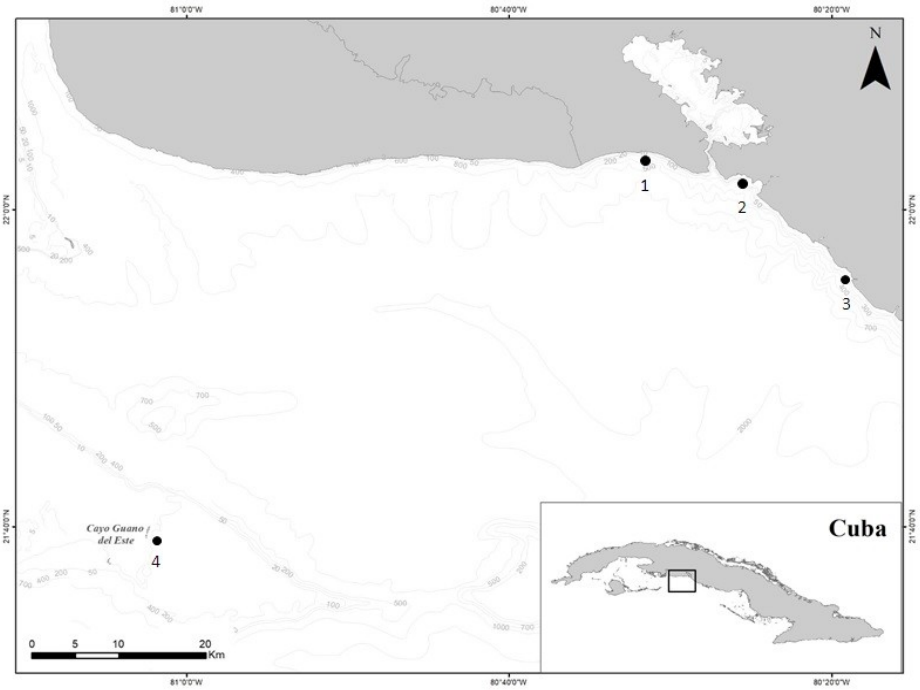

Figure 1. Sampling sites on the central-southern region of Cuba. 1: Mangles Altos (22°03′19″N 80°30′16″W), 2: Delfinario (22°02′16″N 80°25′45″W), 3: Guajimico (21°55′38″N 80°18′36″W) and 4: Cayo Guano del Este (21°40′13″N 81°02′18″W).

number of cells initially isolated in each sampling campaign.

Growth of Gambierdiscus/Fukuyoa species: Glass Erlenmeyer flasks of 500 mL in triplicate were used for the study of growth curves of *Gambierdiscus*/*Fukuyoa* strains, in which 200 mL of f10k medium was inoculated at a density of approximately 50 cells mL^{-1} (Caillaud *et al.* 2011). Erlenmeyer flasks were placed in a culture chamber (EGCS 301, EQUITEC) under the same light and temperature conditions. Every three or four days over a period of 44 days, 3 aliquots of 0.5-1 mL were taken from each replicate culture for cell counting using a Sedgewick-Rafter chamber under an inverted microscope (Axiovert 40 CFL, Zeiss) at X100 final magnification. The aliquots were taken under sterile conditions homogenizing previously the culture with gentle and circular-horizontal movements. Before counting (in triplicate), the samples were diluted with filtered seawater and fixed in acid Lugol's solution. The concentration of cells was calculated averaging of three cell counts and three replicate aliquots.

The specific growth rate (μ; expressed in $days^{-1}$) for each *Gambierdiscus*/*Fukuyoa* species was calculated using the slope of a growth curve during the exponential growth phase according to the following formula, $\mu=\ln (N_2-N_1)/t_2-t_1$ (Guillard 1973) where N corresponds to the cell numbers and t to the culture time, in days. The exponential growth phase was determined by plotting ln cell number versus time. The doubling time (T_2; expressed in days), the time between two successive cell divisions, was calculated as $T_2=\ln 2/\mu$. The number of cell divisions per day was determined by the reciprocal of doubling time. The productivity of each culture was calculated taking into account the maximum cell density and the total volume of culture medium used (200 mL). Significant differences between cultured species for each growth measurement (specific growth rate, doubling time, number of cell divisions per day and maximum cell density) were assessed with one-way ANOVA using Statistica 7.0. Differences between samples were considered significant at $p < 0.05$.

Results

Success rate of the isolations: Details about the sampling and isolation campaigns at the corresponding places are showed in Table I. With the exception of the sampling campaign carried out in 02/2018 when the isolations started right after sampling, there was a delay between sampling and isolation of at least two weeks. There was a maximum delay of two months for the sampling campaign carried out in Cayo Guano del Este.

The success rate of cell isolation ranged between 0 to 29.7 %. No successful culture was obtained from the site Delfinario in either of the two sampling/isolation campaigns done. There was no correlation between the isolation delay and the success rates. The highest success rate corresponded to the sample with *G. caribaeus* and other unidentified species. The rates in samples with *G. carolinianus* averaged 9 % success. Only three *Gambierdiscus*/*Fukuyoa* species were successfully cultured: *G. belizeanus*, *G. caribaeus* and *G. carolinianus*.

Growth curves of Gambierdiscus/Fukuyoa species: The growth rate varied from 0.14 $days^{-1}$ for both *Gambierdiscus belizeanus* and *Gambierdiscus carolinianus* to 0.17 $days^{-1}$ for *Gambierdiscus caribaeus*. The number of cell divisions per day ranged from 0.20 for *G. carolinianus* to 0.25 for *G.*

Table I. Cell isolation details of *Gambierdiscus/Fukuyoa* species.

Sampling campaign	Isolation campaign	Sampling place	No. of isolated cells	Number of successful cultures	Success rate (%)
02/2016	03/2016	Mangles Altos	166	13 cultures, all identified as *G. carolinianus*	7.8
02/2016	03/2016	Delfinario	29	0	0
12/2016	02/2017	Cayo Guano	75	1 culture identified as *G. belizeanus*	1.3
02/2017	02/2017	Guajimico	37	11 cultures, 5 identified as *G. caribaeus* and 6 unidentified	29.7
02/2017	02/2017	Delfinario	15	0	0
02/2018	02/2018	Mangles Altos	30	3 cultures, all identified as *G. carolinianus*	10

caribaeus. Conversely, the doubling time values for the three species ranged from 3.9 days for *G. caribaeus* to 4.9 days for *G. carolinianus* (Table II). There were no significant differences for the growth rate, number of cell divisions per day and doubling time between the three *Gambierdiscus* species.

However, there were significant differences for the maximum cell density among *Gambierdiscus* species ($p=0.024$, $F=8.609$). The highest values of maximum cell density and productivity (3242 cells mL^{-1} and 648 400 cells, respectively) were recorded for the *Gambierdiscus belizeanus* strain on the 29th day of the batch culture, while the *Gambierdiscus caribaeus* strain showed the lowest values (731 cells mL^{-1} and 146 200 cells, respectively) recorded on the 33th day of the culture (Fig. 2).

Discussion

Despite the fact that *Gambierdiscus*/*Fukuyoa* genera have been extensively used in several research topics such as physiology and toxin production (Chinain *et al*. 2010, Kibler *et al*. 2012, Tester *et al*. 2013, Xu *et al*. 2016) they are considered to be among the most difficult benthic photosynthetic dinoflagellates to be cultured (Assunção *et al*. 2019).

Recently basic methodological details for the successful establishment of cultured strains have been available (Assunção *et al*. 2019); however quantitative information about success in culture establishment for these genera have seldom been published. In a recent paper Lozano-Duque *et al.* (2018) reported a culture success rate of 43 %. In our study the success rate of isolation varied between 0 to 29.7 %. The low rate recorded during the sampling campaign carried out in Cayo Guano del Este suggest that very long time lapse between sampling and isolation seems to affect considerably the success rate of isolations. The absence of successful cultures from the site Delfinario could be related to the low abundance of cells in these samples decreasing the likelihood to obtain successful cultures.

According to the experience gained in the present research, the correct manipulation of cells and cleaning of the materials used are key factors in the success of the cell isolations. Some measures can be taken such as the decontamination of microscope slides with 70% ethanol allowing the isolation procedure on aseptic surfaces, the use of glass capillary pipettes with the right shape and size that guarantee the integrity of the cell throughout the isolation process (at least 6 steps of pick up and discharge), and the careful cleaning of the micropipettes with sterile seawater between successive transfers of cells to reduce the risk of contamination (Andersen 2005). Additionally, abrupt changes in salinity must be avoided through gradual addition of small volume of fresh medium while increasing the volume of the culture in the multiwell plates. The evaporation of the medium is another factor to consider because this can increase salinity above the upper tolerance limits, specifically when cells are growing in very small volumes in the multiwell plates. When the available plates are not originally designed for microalgae culture, it is convenient to fill the outer wells of the plate with sterile medium to avoid evaporation when the sealing with parafilm is not enough.

Seven *Gambierdiscus* or *Fukuyoa* species have been identified in the south central region of Cuba using molecular screening techniques (Díaz-Asencio *et al.* 2019a; Díaz-Asencio *et al.* 2019b). However only three *Gambierdiscus* species (*G. belizeanus*, *G. caribaeus* and *G. carolinianus*) were successfully isolated and cultured (Litaker *et al.* 2010, Tester *et al.* 2013). These three species were indeed the most predominant species in the south central region of Cuba which could explain that statistically they would be more often isolated. Time lapse between sampling and isolation could explain differences in success among species since certain species could resist better than others in the bottle while some could even continue to divide. Additionally, isolation procedures and specific

Table II. Growth parameters for the cultured *Gambierdiscus* species from central-southern Cuba.

***Gambierdiscus* species**	**Specific growth rate ± SEM ($days^{-1}$)**	**Doubling time (days)**	**Cell divisions per day**	**Maximum cell density ± SEM ($cells\ mL^{-1}$)**
Gambierdiscus caribaeus	0.17 ± 0.05	3.9	0.25	731 ± 238
Gambierdiscus carolinianus	0.14 ± 0.01	4.9	0.20	1554 ± 112
Gambierdiscus belizeanus	0.14 ± 0.02	4.8	0.21	3242 ± 868

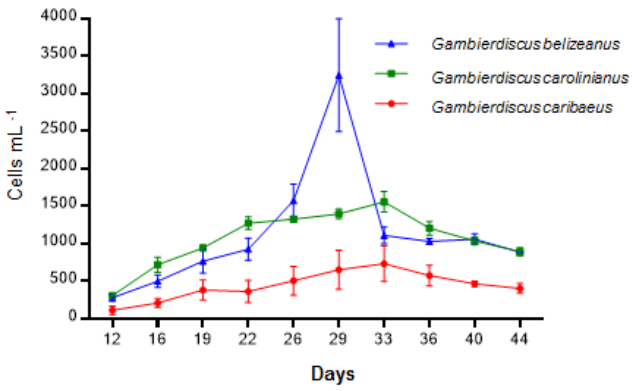

Figure 2. Growth curves for *Gambierdiscus* species grown statically in enriched natural seawater with f10k medium. Each point represents the average ± SEM of three experiments

culture conditions could have been more favorable to these three species indicating their higher robustness to grow in culture.

The specific growth rates for the three *Gambierdiscus* species between 0.14-0.17 day^{-1} were in the range obtained by Litaker *et al.* (2017) using similar environmental culture conditions. However, highest growth rates have been reported previously for these species using specific conditions mainly regarding temperature. For example, Kibler *et al*. (2012) described a maximum growth rate of 0.20 day^{-1} for *G. belizeanus* at 28 °C and 0.35 day^{-1} for *G. caribaeus* at 31 °C. Specifically for *G. caribaeus* the temperature used in our study was out of its optimum growth range between 29.2 and 32.4 °C. After Tester *et al*. (2010) *G. belizeanus* and *G. caribaeus* recorded their maximum growth rates above 29 °C. Conversely, the salinity of 32 seems to fit well with the growth optimal requirement for the three species studied. Kibler *et al.* (2012) showed a broad optimum growth range for all the species tested including *G. belizeanus*, *G. caribaeus* and *G. carolinianus*.

This could partially explain the significant differences found for the maximum cell density among species and the lowest value *G. caribaeus* showed. On the contrary *G. belizeanus* showed a high peak consistent within replicates cultures what indicated its better adaptation to the culture medium used. However, the three *Gambierdiscus* species reached their maximum around the same time (30 days) and showed no clear stationary phase nor a typical growth curve, which could be related to the slow growth obtained. Variations in the chemical composition of the culture medium during growth curve studies of Cuban *Gambierdiscus* strains should be further investigated since it is known that their growth is also likely to be influenced by other parameters which are still poorly studied (Assunção *et al*. 2019).

Among these chemical parameters include pH changes through decrease of CO_2 concentration as cell mass increase (Dixon & Syrett 1988). The nutrient concentrations also affected the *Gambierdiscus* species productivity in culture. Although species in the *Gambierdiscus* genus are oligotrophic organisms, it is known that after approximately three weeks of culture, the progressive accumulation of microalgal biomass decreases the availability of nutrients and light which could makes slower dinoflagellate growth (García 2012). On the other hand, during metabolic activities of microalgae, nutrients could experiment transformations from one stage to another. For example, with the increases of pH in the culture, the ammonium generated during the nitrogen assimilation processes may be transformed in free ammonia which is toxic for microalgae (Borowitzka 1998).

In summary, this study pinpoints the challenges of isolating and culturing *Gambierdiscus* species. Some methodological details for the successful establishment of new cultures were discussed. Three *Gambierdiscus* species (*G. belizeanus*, *G. caribaeus* and *G. carolinianus*) were isolated and their growth curve investigated. These basic studies on the growth of *Gambierdiscus* species are needed for the ongoing and future toxicological researches in Cuba.

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