CHARACTERIZATION OF STEROLS IN THE DINOFLAGELLATE *GAMBIERDISCUS CAROLINIANUS* AND CHLOROPLAST-BASED GALACTOLIPIDS IN *G. CAROLINIANUS* AND *PYRODINIUM BAHAMENSE*: COMPARISON TO OTHER ARMORED DINOFLAGELLATES

By

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ABSTRACT

This thesis delves into the chemotaxonomy of dinoflagellates. The first aspect of this study focuses on the galactolipids of *Pyrodinium bahamense* and *Gambierdiscus carolinianus*. C_{20}/C_{18} galactolipids were predominant in *G. carolinianus*, just like other armored dinoflagellates. Conversely, *P. bahamense* displayed approximately equal amounts of 18:5/18:4 and 20:5/18:4 monogalactosyldiacylglycerol (DGDG), placing it in an intermediary position between the two clusters. *P. bahamense* also displayed a near near total lack of detectable monogalactosyldiacylglycerol (MGDG) with only trace amounts of 18:5/18:4 and 18:4/18:4 MGDG in some of the isolates. Other dinoflagellates possess more DGDG than MGDG but it was significant. These results were consistent for *P. bahamense* grown under two levels of irradiance (at below saturating intensities). This study presents the first characterization of galactolipid composition in these two species and the first description of a peridinin-containing species generally lacking MGDG. The second aspect of this study is also the first exploration of sterol composition in *G. carolinianus*. Nine sterols were identified, with cholesterol, 24-methylcholesta-5-en-3β-ol, and dinosterol being predominant. Comparative analysis with other armored dinoflagellates revealed shared sterols and distinct abundance patterns, notably a higher presence of 24-methylcholesta-5-en-3β-ol in *G. carolinianus*. This suggests its potential as a chemotaxonomic marker for the species. Overall, the study provides galactolipid profiles for *P. bahamense* and *G. carolinianus* and a sterol profile for *G. carolinianus.*

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1.1 ABSTRACT

Previous examinations using positive-ion electrospray mass spectrometry (ESI/MS) and ESI/MS/MS of the chloroplast-associated galactolipids, mono- and digalactosyldiacylglycerol (MGDG and DGDG, respectively), of photosynthetic, peridinin-containing dinoflagellates have shown that such taxa cluster according to the composition of associated polyunsaturated fatty acids (PUFAs) in the major forms (species) of MGDG and DGDG. Specifically, these dinoflagellates form one cluster characterized by the major presence of the C_{18} PUFAs octadecatetraenoic [18:4(n-3)] and octadecapentaenoic [18:5(n-3)] acid in the *sn*-1 and *sn*-2 positions of the glycerol backbone of MGDG and DGDG, and a second cluster where the C_{20} PUFA eicosapentaenoic [20:5(n-3)] acid is found in the *sn*-1 position and either 18:5(n-3) or 18:4(n-3) is found in the *sn*-2 position. This study focuses on a chemotaxonomic comparison between the galactolipids of toxin-producing *Pyrodinium bahamense* and *Gambierdiscus carolinianus* from North American coastal waters, and previously examined, phylogenetically related, peridinin-containing, armored taxa. Results indicate that *G. carolinianus* is clearly a C_{20}/C_{18} (*sn*-1/*sn*-2) dinoflagellate. In contrast, *P. bahamense* (three isolates examined) is unusual as a peridinin-containing dinoflagellate in two regards. First, while many armored dinoflagellates possess more DGDG than MGDG, it displayed a near total lack of detectable MGDG with only trace amounts of 18:5/18:4 and 18:4/18:4 MGDG in some of the isolates. Second, DGDG was characterized by approximately equal amounts of 18:5/18:4 and 20:5/18:4 DGDG, placing it in an intermediary position between the two clusters described above. These results were consistent for *P. bahamense* grown under two levels of irradiance (at below saturating intensities). Our study presents the first characterization of galactolipid composition in these two species and the first description of a peridinin-containing species generally lacking MGDG.

Keywords: DGDG, Dinoflagellate, Galactolipid, *Gambierdiscus*, MGDG, *Pyrodinium*

Highlights

- Galactolipids of *Gambierdiscus carolinianus* resembled other armored C_{20}/C_{18} cluster dinoflagellates.
- Galactolipids of *Pyrodinium bahamense* were composed almost entirely of digalactosyldiacylglycerol (DGDG).
- *P. bahamense*'s galactolipids were a mixture of C_{20}/C_{18} and C_{18}/C_{18} DGDG.

1.2 INTRODUCTION

Armored dinoflagellates, also known as thecate dinoflagellates, are a group of marine protists that possess a protective covering called an armor or theca (Riding *et al.*, 2023). This theca is composed of cellulose plates, or plates made of a combination of cellulose and other materials like silica or calcium carbonate, and provides rigidity and protection to the cell (Kwok *et al.*, 2023). The theca of armored dinoflagellates varies in shape and complexity across species. It may consist of two overlapping halves or of multiple plates (Mertens *et al.*, 2015) to form a diagnostic pattern for a given dinoflagellate (Hoppenrath, 2017; Dale, 2023).

Some armored dinoflagellates, like *Alexandrium tamarense* (Lebour) Balech, can form harmful algal blooms (HABs), also known as red tides. These blooms can have detrimental effects on marine ecosystems and human activities. HABs can produce toxins that accumulate in shellfish and other organisms, causing harmful effects on marine life and posing risks to human health (Zingone *et al.*, 2021). Armored dinoflagellates are primarily found in marine environments, including both coastal and open ocean waters (Durán-Riveroll *et al.*, 2019). They are an important component of phytoplankton communities, where they contribute to primary production in marine ecosystems, playing a vital role in the food web where they have a role in carbon sequestration and export to deep waters to influence global climate regulation (El Semary, 2022).

Phylogenetics is crucial for understanding the biodiversity and evolutionary processes in different organisms, including dinoflagellates (Orr *et al.*, 2012). It helps identify common ancestry, estimate divergence times, and uncover patterns of speciation and evolutionary adaptations (Prazeres & Renema, 2019). For example, Orr *et al.*'s (2012) research infers that

armored/thecate dinoflagellates, including *Coolia monotis* Meunier*, Alexandrium minutum* Halim*, Gambierdiscus toxicus* R.Adachi & Y.Fukuyo, among other Gonyaulacales, evolved from athecate ancestors. Other armored orders that share this common ancestor include the Dinophysiales, Suessiales, Peridiniales, and Prorocentrales (Orr *et al.*, 2012).

Leaw *et al.* (2005) revealed a close phylogenetic relationship between *Alexandrium* and *Pyrodinium* (of order Gonyaulacales). They also showed that *Coolia* and *Ostreopsis* are very close phylogenetically. *Gambierdiscu*s also has a common ancestor with the immediate ancestor of *Alexandrium* and *Pyrodinium,* according to Leaw *et al.* (2005). Although, based on the research by Gómez *et al.* (2015), the genera of dinoflagellates that are most closely related to *Alexandrium* and *Coolia* are *Gambierdiscus* and *Fukuyoa,* followed by the *Ostreopsis.* These are all known to produce potent toxins that can have severe impacts on marine ecosystems and human health. *Alexandrium* species and *Pyrodinium bahamense* L.Plate, for instance, are responsible for the production of paralytic shellfish toxins (PSTs), which can accumulate in shellfish and cause paralytic shellfish poisoning (PSP) in humans (Montoya *et al.*, 2018). *Coolia* species, on the other hand, produce cooliatoxins, which have been associated with harmful algal blooms (HABs) and fish kills (Marampouti *et al.*, 2021). *Gambierdiscus* is notorious for producing ciguatoxins, which can accumulate in fish and cause ciguatera fish poisoning (CFP) in humans (Soliño & Costa, 2020). *Ostreopsis* species produce palytoxin and its analogs, which can be harmful to marine organisms and have been associated with human respiratory issues (Faimali *et al.*, 2012)

Galactolipids are a class of glycolipids that contain a galactose(s) moiety attached to a glycerol backbone at the *sn*-3 position, which is further esterified with two fatty acid chains at the *sn*-1 and *sn*-2 positions (Dörmann, 2020). Monogalactosyldiacylglycerol (MGDG) and

digalactosyldiacylglycerol (DGDG) are common in peridinin-containing dinoflagellates (Gray *et al.*, 2009b). Gray *et al.* (2009a) also initially identified trigalactosyldiacylglycerol (TGDG) in a *Gymnodinium* sp., although this galactolipid form is generally rare in dinoflagellates and its role as a photosynthetically related, chloroplast-associated lipid is unknown. Conversely, MGDG and DGDG play crucial roles in the structure and function of photosynthetic membranes in various organisms, including dinoflagellates, where they are the most abundant lipids in the thylakoid membranes (Kobayashi, 2016). As integral components of the photosynthetic machinery, they form the matrix for the insertion of photosynthetic pigments, such as chlorophylls and carotenoids, allowing for the capture of photons to facilitate their conversion into chemical energy (Fujii *et al.*, 2019). In dinoflagellates, galactolipids are hypothesized to contribute to the fluidity and flexibility of the chloroplast membrane, allowing for efficient membrane dynamics and cellular processes (Lyon & Mock, 2014).

Gray *et al.* (2009b) observed that peridinin-containing, photosynthetic dinoflagellates clustered according to the presence of C_{20} and C_{18} fatty acids as the dominant fatty acids in major forms (species) of MGDG and DGDG, where eicosapentaenoic acid [20:5(n-3)] was the C_{20} fatty acid and octadecapentaenoic [18:5(n-3)] and octadecatetraenoic [18:4(n-3)] were the C_{18} fatty acids (note that the n-3 notation is omitted from hereon for simplicity). Thus, there was one cluster of C_{20}/C_{18} dinoflagellates (with fatty acids listed according to *sn*-1/*sn*-2 regiochemistry) and a separate cluster of C_{18}/C_{18} dinoflagellates. Understanding the chemotaxonomic relationships between these organisms provides insights into the evolution of these ecological traits, allelopathy, and predator-prey interactions (Prazeres & Renema, 2019).

The objective of this study was to evaluate the distribution of the intact forms of MGDG and DGDG in *P. bahamense* and *Gambierdiscus carolinianus* Litaker, Vandersea, M.A.Faust,

Kibler, W.C.Holland & P.A.Tester as they are phylogenetically close to toxin-producing *Alexandrium* and *Coolia.* Both species are environmentally relevant toxin-producers, with *P. bahamense* being found in Florida waters (Lopez *et al.,* 2021b), such as Tampa Bay and Indian River Lagoon, and *G. carolinianus* being found within the Gulf of Mexico (Núñez-Vázquez *et al.,* 2018). Using a North Carolina isolate of *G. carolinianus*, we demonstrate that the galactolipids of *G. carolinianus* greatly resemble those of phylogenetically related *C. monotis* in being found within the C_{20}/C_{18} cluster of peridinin-containing dinoflagellates. However, the galactolipids of Florida isolates of *P. bahamense* are exceptional in that, while resembling the mixture of C_{20}/C_{18} and C_{18}/C_{18} forms of DGDG as previously observed in *A. tamarense*, they are mostly devoid of MGDG.

1.3 MATERIALS AND METHODS

Cultures Growth and Cell Harvesting

G. carolinianus (ARC 160) was acquired from Algal Resources Collection (Wilmington, NC, USA) and was grown in 2 L of L1-Si medium (Guillard & Hargraves, 1993) in 2.8 L Fernbach flasks at Middle Tennessee State University. The growth medium was prepared and distributed using 1 µm-filtered seawater from the Gulf of Mexico. Salt (NaCl) was added to the bottles until the salinity measured 35 psu prior to autoclaving. *G. carolinianus* was grown in duplicate at 20ºC and irradiance of approximately 50 µmol photons $m^{-2}s^{-1}$ using a combination of cool white fluorescent and LED bulbs on a 14:10 light:dark cycle for approximately 6 months as an exceptionally slow growing culture. Cell counts were not obtained because the cells accumulated as a biofilm at the bottom of each flask.

P. bahamense isolates from the Indian River Lagoon, FL (CCFWC 1003) and Tampa Bay, FL (CCFWC 1004-1005) were maintained at the Florida Fish and Wildlife Conservation Commission (FWC, St. Petersburg, FL). Isolates were grown in duplicate in sterile vented polystyrene cell culture flasks at 26ºC in approximately 1.3 L of autoclaved and sterile (0.2 µm)-filtered Tampa Bay water (25 psu) enriched with GSe/4 nutrients (Doblin *et al.,* 1999). Irradiance was provided by cool white fluorescent bulbs on a 14:10 light:dark cycle with irradiance approximately 80 µmol photons $m^{-2}s^{-1}$ (or 50 µmol photons $m^{-2}s^{-1}$ while creating a lower light scenario); for the effect of irradiation on galactolipid composition experiment described in the Results, cells were grown in triplicate. Cells were harvested when abundance reached approximately 5,000 cells/mL.

Cells were separated from the media via vacuum filtration. During this process, the dinoflagellate cells were collected on 1 μm pore size Whatman 934-AH glass microfiber filters (GE Healthcare, Chicago, Illinois, USA), and the filters were stored at -80ºC until lipid processing. For the *P. bahamense* lipids, the filters were stored at -80ºC until they were shipped overnight on dry ice from St. Petersburg, FL to Murfreesboro, TN, and upon receipt were placed at -80ºC until lipid processing.

Lipid processing

Total lipids were extracted using a modified Bligh and Dyer method (Leblond & Chapman, 2000), and were separated into 5 different fractions according to polarity using column chromatography and solvents with varying polarity. Hence, with each fraction, the polarity of the collected lipids increased. Fraction 1 used 12 mL of methylene chloride to collect the most non-polar lipids, including hydrocarbons and sterol esters. For fraction 2, 15 mL of 5% acetone in methylene chloride with 0.05% acetic acid was used to collect free sterols and triglycerides. Fraction 3 used 10 mL of 20% acetone in methylene chloride to collect monoacylglycerols. For fraction 4, 45 mL of acetone was used to collect glycolipids (e.g., MGDG, DGDG). Finally, fraction 5 used 15 mL of methanol with 0.1% glacial acetic acid to collect the most polar lipids, such as phospholipids and betaine lipids (Leblond & Chapman 2000). The vials containing each fraction were stored at -20°C until further processing.

Analysis of intact forms of MGDG and DGDG

The glycolipid fraction (fraction 4) was dissolved in a solution of methanol, chloroform, and 50 mM sodium acetate according to Welti *et al.* (2002) to produce positively charged sodium adducts [$M+Na^+$]. These adducts were subjected to a positive-ion electrospray/mass spectrometry (ESI/MS) scan from *m/z* 100-2,000 via direct injection of a 5 μl sample volume

into a methylene chloride carrier solvent at 0.5 ml min^{-1} into a Finnigan DecaXP ion trap mass spectrometer (Waltham, MA, USA) according to the procedure of Gray *et al.* (2009b). Assessment of detection limits for MGDG and DGDG, and amount per *P. bahamense* cell, was done using purified MGDG and DGDG standards from Matreya (State College, PA, USA). In order to verify that any galactolipids were found only in the glycolipid fraction, fractions 3 and 5 were also examined via positive-ion ESI/MS; no galactolipids were found in these fractions.

Galactolipids were then subjected to subsequent ESI/MS/MS analysis, employing a collision energy range of 37.5 to 48%. The identification of significant fatty acids involved comparing the masses of the intact ions with those of their fragmented counterparts. To determine the positions of the acyl chains (*sn*-1 or *sn*-2), a modified version of the method established by Guella *et al.* (2003), as described by Gray *et al.* (2009b) was employed.

Fatty acids associated with the galactolipid-containing fraction were examined via gas chromatography/mass spectrometry (GC/MS) as fatty acid methyl esters (FAMEs) and 4,4-dimethyloxazoline (DMOX) derivatives according to Leblond & Chapman (2000) using the instrument conditions of Leblond *et al.* (2019).

Statistical treatment of data

The means of galactolipid relative percentages at different light intensities were compared using a two-tailed unpaired t-test with Welch's correction, using GraphPad Prism v9.5.0 (GraphPad Software Inc., San Diego, CA, USA).

1.4 RESULTS

Table 3: Relative abundance (in % of total fragment height using listed masses) of galactolipids as determined via positive-ion ESI/MS and ESI/MS/MS. Data are listed for duplicate cultures.

		Pyrodinium bahamense (CCFWC Isolates)						*Gambierdiscus carolinianus*	
Galactolipid $[M+Na]^+$	Mass[1]	1003A	1003B	1004A	1004B	1005A	1005B	ARC160A	ARC160B
18:1/16:0 MGDG	779							8.0	0.09
18:5/18:4 MGDG	791			0.03		0.06	0.003		
18:4/18:4 MGDG	793			0.03		0.05	0.003		
20:5/18:5 MGDG	817							55.8	11.8
20:5/18:4 MGDG	819							15.2	63.1
18:5/18:4 DGDG	953	41.4	36.7	48	48	62.1	56.9		
20:5/18:5 DGDG	979	2.7	2.8			0.02		13.7	15.6
20:5/18:4 DGDG	981	54.6	59.1	49.8	50.2	37.8	43	7.2	9.4
22:6/18:4 DGDG	1007	1.1	1.3	2.2	1.7				

[1]Mass rounded down to nearest odd number for the purpose of simplification.

As shown in Table 1, nine galactolipids were detected overall in the three isolates of *P. bahamense* (CCFWC 1003-1005) and *G. carolinianus* (ARC 160). Four of these galactolipids were forms of DGDG, while the other five were forms of MGDG. The galactolipids of *G. carolinianus* resembled those of other armored C_{20}/C_{18} cluster dinoflagellates (discussed further below), as evidenced by prominent galactolipids at *m/z* 817, 819, 979, and 981 (Fig. 1a) which corresponded to 20:5/18:5 and 20:5/18:4 MGDG, and 20:5/18:5 and 20:5/18:4 DGDG, respectively (Table 1). Also present was 18:1/16:0 MGDG (*m/z* 779) at lesser amounts in the duplicate samples. All polyunsaturated fatty acids (PUFAs) in *G. carolinianus* (and *P. bahamense*, described below) were found to have n-3 positioning of unsaturations (data not shown). In *G. carolinianus*, the 18:1 fatty acid in 18:1/16:0 was octadecenoic acid [18:1(n-9), data not shown].

The galactolipids of all three stains of *P. bahamense* were characterized by two traits. First, with the exception of very low amounts of 18:5/18:4 and 18:4/18:4 MGDG (*m/z* 791 and 793, respectively) in one replicate of isolate CCFWC 1004 and both replicates of isolate CCFWC 1005, MGDG was entirely absent.

Second, the predominant forms of DGDG in the three stains of *P. bahamense* were a mixture of 18:5/18:4 (*m/z* 953) and 20:5/18:4 DGDG (*m/z* 981), among lesser amounts of 20:5/18:5 (*m/z* 979) and 22:6/18:4 DGDG (*m/z* 1007, Fig. 1b, Table 1). As an example of how positive-ion ESI/MS/MS was used to assign fatty acids with regiochemical specificity, Fig. 1 also shows mass spectra for 18:5/18:4 and 20:5/18:4 DGDG. In Fig. 1c, the larger *m/z* 679 fragment indicates preferential loss of the 18:5 fatty acid from the *sn*-1 position, while the lesser *m/z* 677 fragment indicates loss of the 18:4 fatty acid from the *sn*-2 position of 18:5/18:4 DGDG. Likewise, in Fig. 1d the larger *m/z* 679 fragment indicates preferential loss of the 20:5 fatty

acid from the *sn*-1 position, while the lesser *m/z* 705 fragment indicates loss of the 18:4 fatty acid from the *sn*-2 position of 20:5/18:4 DGDG.

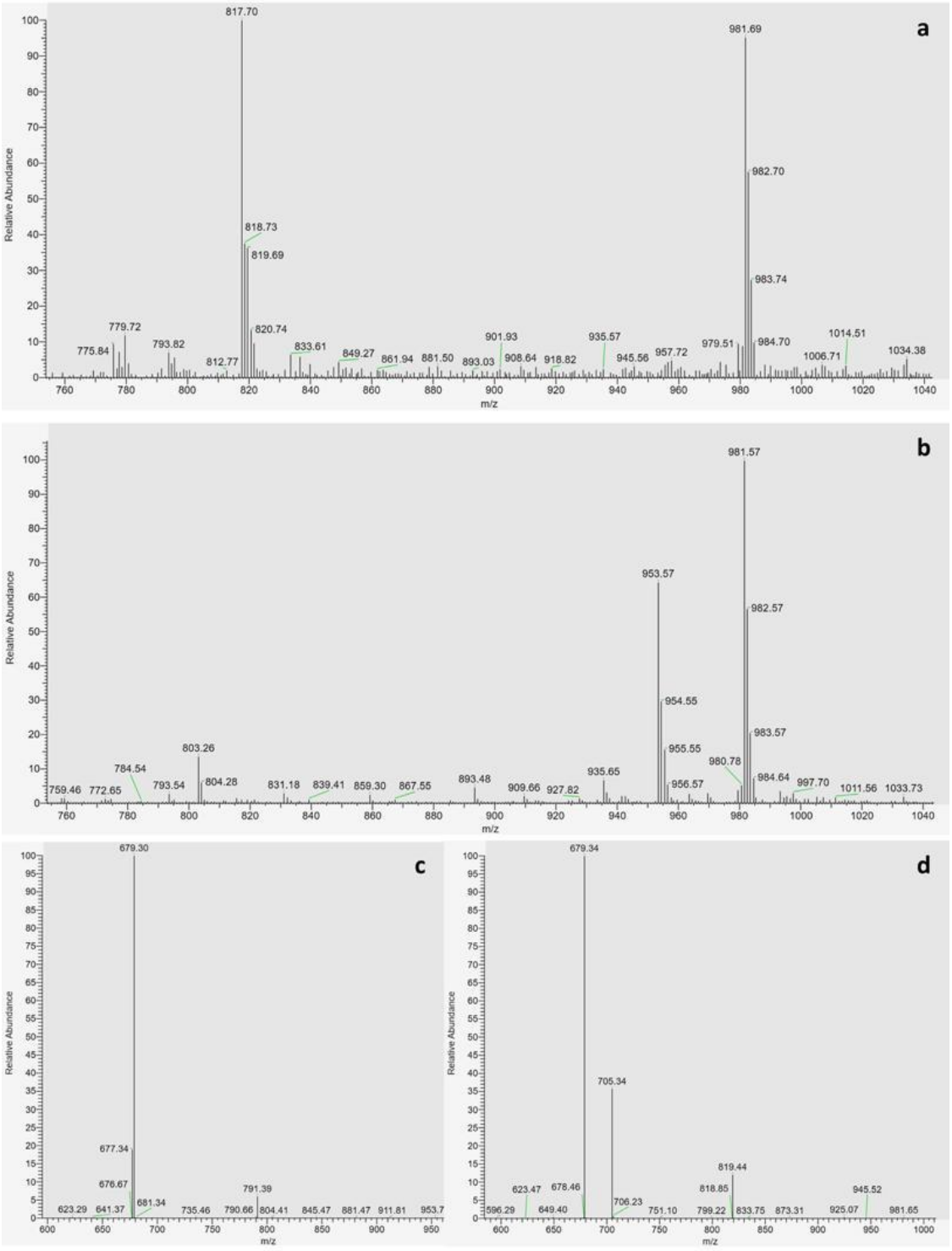

Figure 2: Close-up view of positive-ion electrospray ionization/mass spectrometry (ESI/MS) full -scan spectrum of sodium adducts of galactolipids from (a) *Gambierdiscus*

carolinianus ARC 160 and (b) *Pyrodinium bahamense* CCFWC 1003; major ion masses (m/z values) correspond to galactolipids listed in Table 1. Panels c and d show positive-ion ESI/MS/MS of 18:5/18:4 DGDG (m/z 953) and 20:5/18:4 DGDG (m/z 981), respectively, from *P. bahamense* CCFWC 1003.

To determine if light intensity affects the galactolipid composition as an examination of an environmental condition that can fluctuate under natural growth conditions, and for which there are limited data on its possible effect on galactolipid composition, a follow-on experiment was conducted where *P. bahamense* (CCFWC 1004) was grown in triplicate under two light conditions (50 µmol photons $m^{-2}s^{-1}$ and 80 µmol photons $m^{-2}s^{-1}$). When grown at 50 µmol photons $m^{-2}s^{-1}$, galactolipid composition in *P. bahamense* isolate 1004 was predominately the DGDG forms 18:5/18:4 (33.0 ± 4.6%) and 20:5/18:4 (61.0 ± 7.6%) with form 20:5/18:5 comprising only 2.8 ± 1.6%. The same forms (18:5/18:4 and 20:5/18:4) also dominated when this isolate was grown at 80 µmol photons $m^{-2}s^{-1}$, but the relative abundance for 18:5/18:4 was higher (53.7 ± 3.5%, P-value 0.004) under this light condition compared to lower light, see Table 2.

Table 4: Relative abundance (mean ± standard derivation) of galactolipids grown in low light intensity (50 µmol photons $m^{-2}s^{-1}$) and high light intensity (80 µmol photons $m^{-2}s^{-1}$). Data are listed for triplicate cultures.

Pyrodininum bahamense (CCFWC1004)				
Galactolipid $[M+Na]^+$	Mass[1]	P-value	80 µmol photons $m^{-2}s^{-1}$	50 µmol photons m^2s^{-1}
18:5/18:4 DGDG	953	0.004	53.7 ± 3.5	33 ± 4.6
20:5/18:5 DGDG	979	0.093	0.000 ± 0.00	2.8 ± 1.6
20:5/18:4 DGDG	981	0.060	46.3 ± 3.5	61 ± 7.6

[1]Mass rounded down to nearest odd number for the purpose of simplification

Table 5: Relative abundance (%) of major MGDG and DGDG in previously studied dinoflagellate that are phylogenetically close to *Pyrodinium bahamense* and *Gambierdiscus carolinianus.*

Organism	18:5/18:5 MGDG	18:5/18:4 MGDG	18:5/18:4 DGDG	18:4/18:4 DGDG	20:5/18:5 MGDG	20:5/18:4 MGDG	20:5/18:5 DGDG	20:5/18:4 DGDG
Alex. andersoni CCMP 1718		7.2	22.2	5.5	7.5	16.6	8.5	32.6
Alex. minutum CCMP 113	48.1	11.9	33.8	6.2				
Alex. tamarense UTEX 2521	4.6	4.8	26.5	8.4	5.0	7.6	5.3	37.6
Cool. monotis CCMP 1345					15.8	13.2	3.7	67.3
Coolia sp. EPA					14.8	14.8		70.5
Frag. sp. CCMP 1920			43.1	6.9	19.2	3.2	16.5	11.1
Ling. polyedrum CCMP 1738		4.3			13.2	16.4	54.8	11.4
Pyro. lunula UTEX 2271					14.1			86.0

Source: (Gray *et al.*, 2009b).

1.5 DISCUSSION

Gray et al. (2009b) described galactolipid compositions of armored dinoflagellates phylogenetically related to *P. bahamense* and *G. carolinianus*, and the relative percentages of all galactolipids from this previous work are shown in Table 3. These data reveal that while in all the organisms listed, DGDG is more abundant than MGDG, the additive relative percentage of all forms MGDG in any of these dinoflagellates is never below approximately 15-20% of the total relative percentage of all galactolipids. This observation is also generally true for other, less phylogenetically related, peridinin-containing dinoflagellates, whether they be in the C_{20}/C_{18} or C_{18}/C_{18} cluster, examined by Gray *et al.* (2009b).

C. monotis, *Coolia* sp., and *P. lunula* are clearly C_{20}/C_{18} cluster dinoflagellates per Gray *et al.* (2009b), while *Alexanderium andersonii* Balech, *A. tamarense*, *Fragilidium* sp., and *Lingulodinium polyedrum* (Stein) Dodge are "chimeric" in that they display both C_{20}/C_{18} and C_{18}/C_{18} forms of MGDG and DGDG. However, in the original Gray *et al.* (2009b) paper, all of these species except for *Fragilidium* sp. were clustered with C_{20}/C_{18} dinoflagellates due to higher relative percentages of C_{20}/C_{18} galactolipids. Note that Gray *et al.* (2009b) also observed this "chimeric" mixture of C_{20}/C_{18} and C_{18}/C_{18} galactolipid forms sporadically in a few other less related peridinin-containing taxa; this has been expanded upon in a more recent study on *Amphidinium* (described below). Note that a more expansive examination of the genus *Pyrocystis* was performed by Leblond *et al.* (2010), where one additional form each of MGDG and DGDG was found, as well as two forms of TGDG; nevertheless, the study demonstrated that there is typically much more DGDG than MGDG present within the genus. In our current work, *G. carolinianus* is a clear C_{20}/C_{18} cluster dinoflagellate because of its C_{20}/C_{18} forms of MGDG and DGDG (Table 1).

There was an approximately equal split between C_{20}/C_{18} and C_{18}/C_{18} forms of DGDG in *P. bahamense* (Table 1). However, *P. bahamense* is unusual in that unlike the galactolipids of armored dinoflagellates characterized by Gray *et al.* (2009b) and Leblond *et al.* (2010), those of *P. bahamense* were characterized by near absence of MGDG and near totality of DGDG (Table 1) – such an absence of MGDG was also not observed in the study of galactolipids of freshwater dinoflagellates (some of which are armored) by Anesi *et al.* (2016); this work is a comparable study in terms of the methodology used to identify the composition of intact dinoflagellate galactolipids. Our detection limits for MGDG and DGDG via positive-ion ESI/MS were approximately 0.5 mg/L (ppm) for MGDG and DGDG. Thus, it is possible that MGDG was present at even lower relative percentages (or actual amounts) than shown for isolates 1004 and 1005 (Table 1) where the actual amounts of DGDG in all three isolates, by comparison, were approximately 1 pg/cell – this concentration corresponds to that observed in *A. tamarense* by Leblond & Dahmen (2012).

DGDG is formed by the addition of galactose to MGDG in a reaction catalyzed by DGDG synthase (Zhang, 2020); this may explain why 18:5/18:4 MGDG and 18:4/18:4 MGDG are found in very low (i.e., trace) amounts in some isolates of *P. bahamense*, presumably as putative biosynthetic precursors to both C_{18}/C_{18} and C_{20}/C_{18} forms of DGDG. Our laboratory has examined the galactolipids of several other groups of algae, including chlorarachniophytes (Leblond & Roche, 2009), chromerids (Dahmen *et al.*, 2013; Khadka *et al.*, 2014), diatoms (Dodson *et al.*, 2013), euglinids (Craig et al., 2015), glaucocystophytes (Leblond *et al.*, 2010), raphidophytes (Roche & Leblond, 2011), red algae (Carter & Leblond, 2018), and aberrant plastid (i.e., non-peridinin-containing) dinoflagellates (Leblond & Lasiter, 2009; Leblond *et al.*, 2019, 2023; Graeff *et al.*, 2021). In none have we found an alga that has such a paucity of

MGDG as in *P. bahamense*. In all of these groups of algae, most of which do not include model taxa, it is assumed that MGDG plays the same role that it does in plants, such as the model plant *Arabidopsis thaliana*, where it is involved in a variety of processes related to photosynthesis, such as photoprotection and thylakoid membrane energization (*cf.* Aronsson, 2008; Aronsson *et al.*, 2008). Deficiency of MGDG in plants and model algae leads to increased sensitivity to environmental stresses like high temperature, high light, oxidative stress and impaired photosynthesis (Khozin-Goldberg, 2016). The growth conditions of *P. bahamense* in our experiments were similar to those used to maintain cultures of this alga and were within the range of conditions encountered in nature during blooms, although temperature and light conditions were likely at the lower end of optimal (Lopez *et al.,* 2021a, 2021b). They were also comparable to the growth conditions of the other groups of algae listed above.

The near-total presence of DGDG indicates that in *P. bahamense* it can serve as the chief galactolipid in photosynthetic membranes (Table 1). Like MGDG, DGDG is a major lipid component of thylakoid membranes, which are the sites of photosynthesis in plants, algae, and cyanobacteria (Yoshihara & Kobayashi, 2022). DGDG molecules contribute to the structural organization of thylakoid membranes by forming lipid bilayers (Yoshihara & Kobayashi, 2022). This lipid bilayer arrangement provides a stable environment for the assembly and positioning of photosynthetic protein complexes, such as photosystems I and II, cytochrome b6f complex, and ATP synthase. DGDG also contributes to the stability and integrity of the photosynthetic membrane. Its presence helps to prevent the aggregation and denaturation of membrane proteins under various environmental conditions (Challabathula *et al.*, 2016). DGDG molecules act as a matrix for embedding protein complexes, shielding them from deleterious interactions and maintaining their proper orientation and functionality (Challabathula *et al.*, 2016). Additionally, DGDG modulates the fluidity and flexibility of the

thylakoid membrane. It can reduce the rigidity of the membrane, allowing for efficient diffusion and movement of lipids and proteins within the membrane (Wilhelm *et al.*, 2020). Furthermore, DGDG plays a role in the stability and optimal functioning of photosystems. It contributes to the structural integrity of photosystem complexes and helps to maintain their proper arrangement within the thylakoid membrane (Wilhelm *et al.*, 2020).

The C_{18}/C_{18} forms of "chimeric" MGDG in *A. andersonii*, *A. tamarense*, *Fragilidium* sp., and *L. polyedra* are putative biosynthetic precursors to C_{20}/C_{18} forms, with the assumption that any C_{20} fatty acids are formed by elongation of C_{18} fatty acids, yet, unlike *P. bahamense*, both C_{18}/C_{18} and C_{20}/C_{18} forms of MGDG and DGDG in these species exist as stable end products of galactolipid biosynthesis. Such a mixture of C_{20}/C_{18} and C_{18}/C_{18} forms of MGDG and DGDG has also been observed in species of the unarmored dinoflagellate genus *Amphidinium* which is not phylogenetically close to the armored taxa described above (Leblond *et al.*, 2023 and references therein), and it has been hypothesized there that, while the more prevalent C_{20}/C_{18} forms appear to be more ancestral in terms of dinoflagellate evolution than C_{18}/C_{18} forms because of the putative ancestral position of *Amphidinium* compared to other peridinin-containing dinoflagellates, a C_{18}/C_{18} form(s) is a necessary biosynthetic precursor to C_{20}/C_{18} forms of MGDG and DGDG.

DGDG, as a photosynthetically important galactolipid, has been implicated in the response of photosynthetic organisms to various environmental stresses. Under adverse conditions, such as high light intensity, extreme temperatures, or nutrient limitations, the composition and abundance of DGDG in the membrane can be altered (Du *et al.*, 2018). These changes in DGDG levels can influence the overall lipid composition and properties of the membrane, enabling the organism to adapt and protect its photosynthetic machinery (Bejaoui *et al*., 2016;

Du *et al.*, 2018; Guo *et al*., 2019). In the case of *P. bahamense*, the organism is able to grow photosynthetically in culture with a near absence of detectable MGDG.

Environmental factors like temperature can affect the MGDG to DGDG ratio and/or the fatty acid compositions of individual galactolipids. For example, Leblond *et al.,* (2010) were able to establish this effect on some *Pyrocystis* spp. isolates. As temperature increased from 15°C to 25°C, the abundance of 20:5/18:5 MGDG and 20:5/18:4 MGDG decreased, while that of 20:5/18:5 DGDG and 20:5/18:4 DGDG increased in *P. lunula* and *P. noctiluca*. Leblond *et al.* (2019) also reported the effect of temperature change from 20°C to 30°C on *Karenia mikimotoi* (Miyake & Kominami ex Oda) Gert Hansen & Ø.Moestrup, where it was observed that modulations did occur in MGDG and DGDG forms containing tetradecanoic (14:0) and hexadecatetraenoic acid [16:4(n-3)] not present in peridinin-containing dinoflagellates such as *Pyrocystis*. Also, Leblond *et al.* (2015) varied the temperature for growing *Symbiodinium microadriaticum*, and studied how it affected the lipid composition using ESI/MS. The results revealed temperature-induced variations in the forms of MGDG and DGDG. At 30°C, there was an increase in 16:0-containing forms of MGDG not found at 20°C. The major MGDG and DGDG forms were 18:4/18:5 MGDG and 18:4/18:4 DGDG, but their proportions varied with temperature. Additionally, the study explored betaine lipids, revealing temperature-dependent changes in diacylglycerylcarboxyhydroxymethylcholine (DGCC) and diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine (DGTS).

Furthermore, Zhukova and Titlyanov (2006) also studied the effect of light intensity on dinoflagellate lipids. They varied light intensity from 2% to 95% of photosynthetically active radiation (PAR). This changed the total fatty acid content per cell from 96-112 pg/cell at 2% PAR to 195-210 pg/cell at 95% PAR. Also, with increasing irradiance, the proportion of saturated fatty acids increased slightly, while the sum of PUFA decreased in both phospholipids

(PL) and triacylglycerols (TAG). Some fatty acids such as 22:6(n-3) and 20:4(n-6), were positively correlated with PAR, indicating their association with photosynthetic processes. However, they did not examine MGDG and DGDG specifically. Thus, the effect of varying irradiance on dinoflagellate galactolipid composition (i.e., which fatty acids are found in MGDG and DGDG) remains not well-studied, and is the rationale behind our experiment on the effect of irradiance on the galactolipid composition of *P. bahamense*.

Florida populations of *P. bahamense* are often observed in estuarine transitions zones and can tolerate a wide range of salinities (10–45 psu; Phlips *et al*., 2006.), although blooms (>100,000 cells/L) typically occur between 18-33 psu (Lopez *et al*., 2021b, Lopez *et al*., 2023) – note that in past studies (e.g., Gray *et al.*, 2009a, 2009b), salinity does not appear to affect galactolipid composition and is not expected to be a factor in this current work. As a tropical species, *P. bahamense* thrives in warm summer temperatures, with most blooms being observed between ~27–33° C (Lopez *et al.,* 2021b). Laboratory studies suggest that *P. bahamense* is not a low-light adapted species (Lopez *et al.,* 2021a), but this species may be able to succeed under a range of light regimes due to its ability to vertically migrate. While the relative composition of galactolipids were similar under the two light conditions in our study, these light levels were below the saturation intensity for growth of *P. bahamense* (~130 μmol photons $m^{-2}s^{-1}$, Lopez *et al.,* 2021a). Future work could explore the effect of saturating light levels on the relative composition of these galactolipids.

These data on *G. carolinianus* and *P. bahamense* expand our general knowledge of the galactolipids of armored dinoflagellates, but, more importantly, identify a peridinin-containing, environmentally relevant dinoflagellate in *P. bahamense* that is exceptional in its near-total lack of MGDG in culture with an approximately equal mixture of C_{20}/C_{18} and C_{18}/C_{18} forms of

DGDG. Future work will attempt to examine bloom samples to see if they agree with these lab data.

Author contributions

J.D. Leblond: original concept, lipid processing and analysis, drafting and editing manuscript; T.J. Busari: culture growth, lipid processing and analysis, drafting and editing manuscript; C.B. Lopez: culture growth, drafting and editing manuscript; B.E. Hollingsworth: lipid processing; I.E. Rippy: lipid processing; S.I. Spurlock: lipid processing.

Sterols of the Ciguatoxin-Producing Dinoflagellate *Gambierdiscus carolinianus* as the First Examination of this Genus: Abundance of the Rare Dinoflagellate Sterol 24-Methylcholesta-5-en-3β-ol as Potential Biomarker

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2.1 ABSTRACT

Dinoflagellates play crucial roles in nutrient cycling, primary production, and the overall health of aquatic environments. Among dinoflagellates, the genus *Gambierdiscus* is of particular interest due to its association with ciguatera fish poisoning. While the toxins have been studied extensively, the sterol composition of *Gambierdiscus* remains unexplored. Sterols are essential in maintaining membrane structure in eukaryotes and have a long history of chemotaxonomic utility in dinoflagellates. In this study, we conducted the first examination of sterols in *Gambierdiscus carolinianus*, comparing them to phylogenetically related armored dinoflagellates. We identified nine sterols, including dehydrocholesterol (cholesta-5,22E-dien-3β-ol), cholesterol (cholest-5-en-3β-ol), dinosterol (4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol) and 24-methylcholesta-5-en-3β-ol, with cholesterol, 24-methylcholesta-5-en-3β-ol and dinosterol being the major sterols. Comparisons with the armored dinoflagellate genera *Alexandrium, Coolia, Gonyaulax, Pyrocystis* and *Pyrodinium* revealed shared sterols, such as cholesterol and dinosterol, yet a much higher abundance of 24-methylcholesta-5-en-3β-ol in *G. carolinianus*, indicating it may serve as a possible biomarker for this species. These findings fill a critical knowledge gap regarding the sterol composition of *Gambierdiscus* and provide insights into the chemotaxonomic relationship of *G. carolinianus* to these armored dinoflagellates.

Keywords

Gambierdiscus, Dinoflagellate, Lipid, Sterol

Highlights

- Nine sterols were identified in *G. carolinianus*.
- G. carolinianus comprise cholesterol and dinosterol, just like the armored dinoflagellate genera (*Alexandrium, Coolia, Gonyaulax, Pyrocystis* and *Pyrodinium)*
- High abundance of 24-methylcholesta-5-en-3β-ol in *G. carolinianus* could mean is is a possible biomarker for this species.

2.2 INTRODUCTION

Dinoflagellates, a large and diverse group of photosynthetic and heterotrophic algae, inhabit marine and freshwater ecosystems worldwide, playing crucial roles in nutrient cycling, primary production, and the overall health of aquatic environments (Skarlato et al. 2018). These microscopic organisms are instrumental in the transfer and recycling of essential elements, helping to regulate nutrient availability and to prevent eutrophication (Thornton 2012). Through photosynthesis, dinoflagellates act as primary producers, driving energy flow within aquatic food webs and sustaining the abundance and diversity of marine life (Wilkins et al. 2013).

Among dinoflagellates, the genus *Gambierdiscus* has garnered attention due to its association with ciguatera fish poisoning. This foodborne illness arises from consuming fish contaminated with toxins produced by *Gambierdiscus* species (Dickey and Plakas 2010). Studying *Gambierdiscus* is crucial not only for understanding its ecological roles but also for addressing potential impacts on human health and marine ecosystems (Wells et al. 2015). The intricate interplay between *Gambierdiscus* toxin production, ecological functions, and overall ecosystem health underscores the importance of further exploration of this genus (Schwartz et al. 2016).

Sterols, a class of lipids, are essential components of cell membranes and play vital roles in cellular processes such as growth, signaling, and membrane fluidity of eukaryotes (Guschina and Harwood 2009). The composition and diversity of sterols within microorganisms can offer valuable insights into their physiological adaptations and potential ecological interactions (Akbar et al. 2022). Beyond their immediate cellular functions, sterols have also emerged as

powerful chemotaxonomic tools used to distinguish and classify microorganisms based on their unique sterol profiles (Fernandes et al. 2020).

However, despite their importance, studies on sterols within the genus *Gambierdiscus,* particularly within environmentally relevant species such as *Gambierdiscus carolinianus* Litaker, Vandersea, M.A.Faust, Kibler, W.C.Holland & P.A.Tester, remain notably scarce. *G. carolinianus* as a producer of ciguatoxins has been observed as one of the predominant *Gambierdiscus* species in waters off the Texas and Louisiana coasts, as well as the broader Gulf of Mexico and Caribbean (Richland et al. 2024; Tester et al. 2013).

Dinoflagellates contain a diverse range of sterols which can be used as complementary data in the dinoflagellate classification process (e.g., Vandergrift et al. 2021). Sterols found in dinoflagellates contain between 27 and 31 carbon atoms (Leblond and Chapman 2002; Volkman 2016). They also have substituted groups and nuclear unsaturations in them at different positions, along with various side chains, to yield an almost unprecedented diversity amongst sterol-producing algae, with dozens of possible sterols (Leblond et al. 2010). Dinosterol (4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol) is established as a major sterol component of many, but not all, dinoflagellates and an important sediment biomarker for marine organisms (Amo et al. 2010) it, along with the presence or absence of cholesterol (cholest-5-en-3β-ol), is also useful for comparisons of chemotaxonomic relationships (Leblond et al. 2010). Some groups of dinoflagellates, such as the Kareniaceae, possess sterols rarely found in other dinoflagellate taxa - see discussion Leblond et al. (2023) on the distribution of the 4α-methyl sterols (24*R*)-4α-methyl-5α-ergosta-8(14),22-dien-3β-ol (gymnodinosterol) and 27-*nor*-(24*R*)-4α-methyl-5α-ergosta-8(14),22-dien-3β-ol (brevesterol) in the Kareniaceae and other gymnodinioid dinoflagellates.

Phylogenetic data reveal that *Alexandrium* species, *Coolia* species and *Pyrodinium bahamense* L. Plate are closest to *Gambierdiscus* (Gómez et al. 2015). Ellegaard et al. (2018) also

established a close phylogenetic relation between certain *Alexandrium* species and *Gonyaulax* species, and Litaker et al. (2009) established a relationship between *G. carolinianus* and *Pyrocystis lunula* Schütt. The present study aims to conduct the first examination of sterols within the genus *Gambierdiscus,* with a specific focus on the potential for a *G. carolinianus* sterol biomarker and chemotaxonomic comparison to the armored genera described above.

2.3 METHODS

Culture growth and cell harvesting: *G. carolinianus* (strain ARC 160), which was originally isolated from Cape Fear waters, North Carolina, USA), was procured from the Algal Resources Collection (Wilmington, North Carolina, USA). The cultivation of *G. carolinianus* was done in 2.8 L Fernbach flasks containing 2 L of L1-Si medium as prescribed by Guillard and Hargraves (1993). The growth medium was prepared and distributed using 1 µm-filtered seawater sourced from the Gulf of Mexico. To attain a salinity level of 35 practical salinity units (psu), sodium chloride (NaCl) was introduced into the containers before subjecting them to autoclaving.

The cultivation of *G. carolinianus* was executed in duplicate under a temperature of 20°C and an approximate irradiance of 50 µmol photons/m²s using a combination of cool white fluorescent and LED bulbs, adhering to a photoperiod of 14 hours light and 10 hours darkness. The cultivation period spanned approximately 6 months due to the notably sluggish growth rate of the culture. Quantitative cell counts were not conducted because the cells amassed as a biofilm, localized at the bottom of each flask.

Cell separation from the culture medium was accomplished through employment of vacuum filtration. Within this procedure, the dinoflagellate cells were amassed onto Whatman 934-AH glass microfiber filters (GE Healthcare, Chicago, Illinois, USA) with a pore size of 1 µm. The ensuing filters were subsequently preserved at a temperature of -80°C until lipid processing.

Lipid processing: The extraction of total lipids was conducted utilizing a modified Bligh and Dyer technique (Leblond and Chapman 2000). Subsequently, these lipids were fractionated into five distinct groups based on their polarity employing column chromatography with solvents of varying polarities.

Consequently, an incremental enhancement in polarity was observed for each successive fraction. Fraction 1 employed 12 mL of methylene chloride to elute the least polar lipids, encompassing hydrocarbons and sterol esters. In the case of fraction 2, a solution comprising 15 mL of 5% acetone in methylene chloride with 0.05% acetic acid was used for the collection of free sterols and triglycerides. Fraction 3 used a solution of 10 mL of 20% acetone in methylene chloride to elute monoacylglycerols. For fraction 4, a quantity of 45 mL of acetone was employed to gather glycolipids. Lastly, fraction 5 involved the use of 15 mL of methanol with 0.1% glacial acetic acid to capture the most polar lipids, such as phospholipids and betaine lipids (Leblond and Chapman 2000). Storage of the vials containing each fraction was undertaken at a temperature of -20°C until further processing.

Sterol analysis: The sterol ester and free sterol fractions were saponified and derivatized to form trimethylsilyl (TMS)-ether derivatives of sterols according to Leblond and Chapman (2002). After saponification, trimethylsilyl ether (TMS) derivatives of sterols were formed with 0.5 ml *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide containing 1% (v/v) trimethylchlorosilane at 80°C for 0.5 h. The reagent was then evaporated under a stream of nitrogen and the derivatives redissolved in 20 µl of methylene chloride prior to analysis.

The derivatives were analyzed via gas chromatography/mass spectrometry (GC/MS) according to Houle et al. (2019) on a Thermo TSQ Quantum XLS (Thermo, Waltham, MA) using an RTX-5MS capillary column (i.e. 30 m length, 0.25-mm inner diam., 0.25-µm film thickness, Restek, Bellefonte, PA) with the following GC/MS conditions: splitless injection with injector was set at 280°C, column was held at 50°C for 1 min, increased to 170°C at 10°C/min, increased to 300°C at 5°C/min, and was held at 300°C for 20 min. The transfer line was set at 275°C, and helium was delivered at a constant velocity of 40 cm/s.

Relative retention times (RRT) to cholesterol were calculated according to the methodology of Jones et al. (1994). Authentic standards of cholesterol, cholestanol, dehydrocholesterol and 24-methylcholesta-5,22E-dien-3β-ol, and 24-methylcholesta-5-en-3β-ol were obtained from Alfa Aesar (Haverhill, MA, USA), SigmaAldrich (St. Louis, MO, USA), Steraloids (Newport, RI, USA), and TCI (Portland, OR, USA) respectively. C24 stereochemistry was not determined in any sterols; this is reflected in Table 1.

2.4 RESULTS AND DISCUSSION

A total of nine sterols were identified in *G. carolinianus* (Table 1). The C_{27} sterols were dehydrocholesterol (cholesta-5,22E-dien-3β-ol, *m/z* 456), cholesterol (*m/z* 458) and cholestanol (*m/z* 460). The C_{28} sterols were comprised of 24-methylcholesta-5,22E-dien-3β-ol (*m/z* 470), 24-methylcholesta-5-en-3β-ol (*m/z* 472), 24-methyl-5α-cholestan-3β-ol (*m/z* 474) and two unidentified C_{28} sterols (*m/z* 470 and *m/z* 472). Also, dinosterol (*m/z* 500), a C_{30} sterol, was present as the only 4α-methyl-substituted sterol. The mass spectra for the three sterols **1-3** were indistinguishable from the mass spectra of authentic standards (not shown); additionally, Fig. 1 shows comparison of sterols **5** and **6** to 24-methylcholesta-5,22-dien-3β-ol and 24-methylcholest-5-en-3β-ol, respectively.

Table 1: Sterols found Sterols found in the free sterol and sterol ester fractions of *Gambierdiscus carolinianus*.

Sterol #	# of Carbons	Suggested Structure (common name where available)	Molecular Weight[a]	Retention Time (min)	RRT[b]	Free Sterols		Sterol Esters	
						Replicate 1	Replicate 2	Replicate 1	Replicate 2
1	C_{27}	Cholesta-5,22E-dien-3β-ol (dehydrocholesterol)	456	36.44	0.856	1.59	1.67	4.64	5.14
2	C_{27}	Cholest-5-en-3β-ol (cholesterol)	458	36.94	1.000	39.24	39.24	31.10	39.51
3	C_{27}	5α-Cholestan-3β-ol (cholestanol)	460	37.07	1.038	1.97	1.45	2.34	2.95
4	C_{28}	Unidentified C_{28} sterol	470	37.18	1.069	Tr	0.50	Tr.	
5	C_{28}	24-Methylcholesta-5,22E-dien-3β-ol[c] (*R*-brassicasterol/*S*-crinosterol)	470	37.45	1.147	3.65	4.92	8.94	10.85
6	C_{28}	24-Methylcholesta-5-en-3β-ol[c] (*R*-campesterol/*S*-dihydrobrassicasterol)	472	38.25	1.378	34.98	37.54	31.48	30.09
7	C_{28}	24-Methyl-5α-cholestan-3β-ol[c] (*R*-campestanol/*S*-dihydrobrassicastanol)	474	38.40	1.421	4.38	2.85	4.54	4.97
8	C_{28}	Unidentified C_{28} sterol	472	38.55	1.464	0.98	0.89	1.87	
9	C_{30}	4α,23,24-Trimethyl-5α-cholest-22E-en-3β-ol (dinosterol)	500	39.68	1.790	13.22	10.93	15.10	6.47
Interfraction comparison of contribution to total sterols as based on peak areas:						52.10	98.50	47.9	1.5

[a]Molecular weight as sterol as a TMS derivative.

[b]Relative retention time to the TMS derivative of cholesterol.

[c]C24 stereochemistry not determined.

Tr - present at less than 0.20%.

Blank spaces - not present.

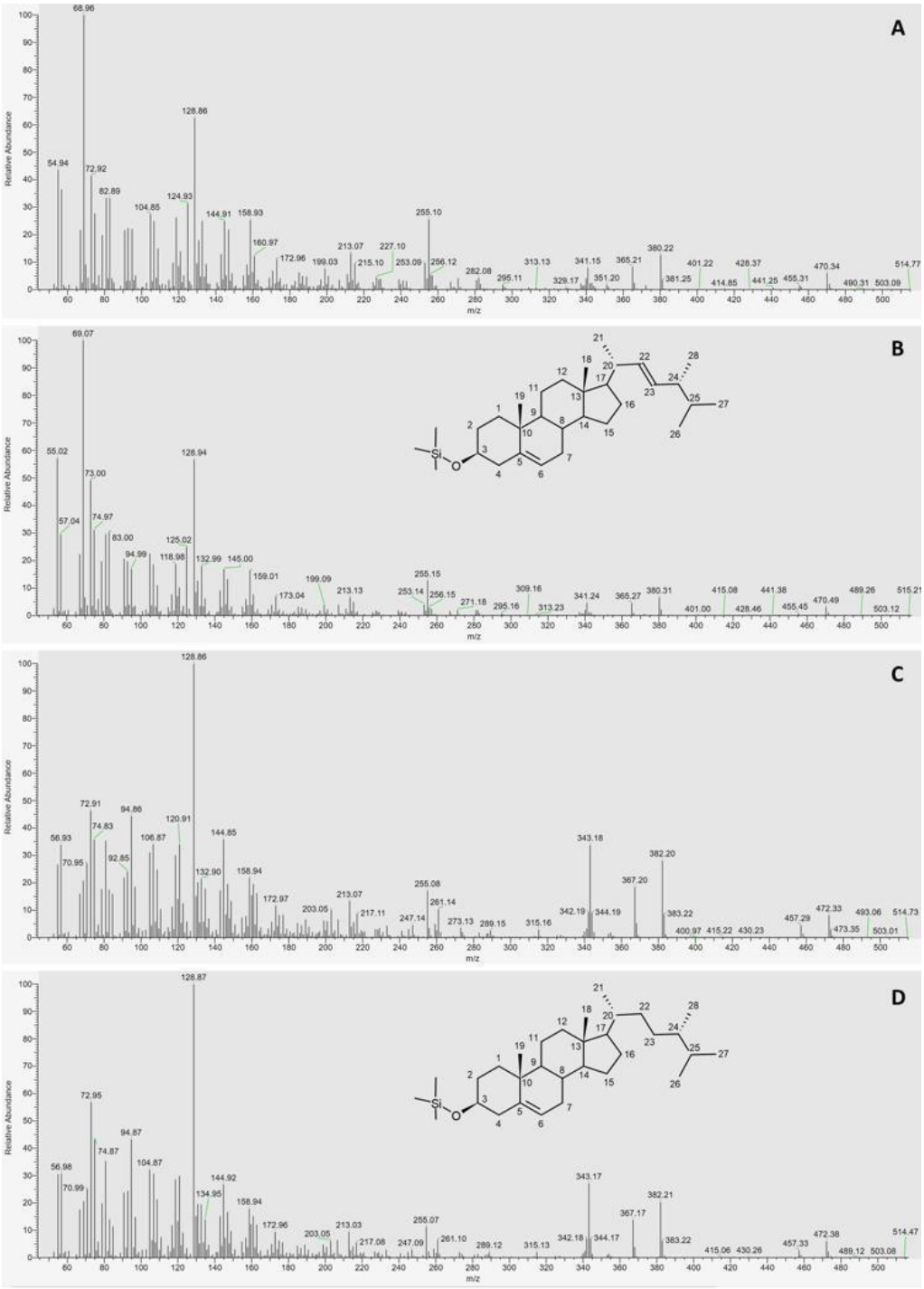

Figure 1: Mass spectra of TMS derivatives of A.) sterol 5 Mass spectra of TMS derivatives of A.) sterol **5** (m/z 470) from *Gambierdiscus carolinianus* ARC 160, B.) authentic 24R-methylcholesta-5,22E-dien-3β-ol (brassicasterol), C.) sterol **6** (m/z 472) from *G. carolinianus,* and D.) authentic 24R-methylcholesta-5-en-3β-ol (campesterol).

While the interfraction comparisons exhibited great variation, the sterol compositions of the replicates were consistent (Table 1); the culture was exceedingly difficult to grow, and we were unable to obtain a third replicate. Notably, cholesterol emerged as the most abundant sterol in both the free sterol and sterol ester fractions. The prevalence of cholesterol in *G. carolinianus* suggests its importance in maintaining membrane integrity and functionality, as observed in other dinoflagellates (Leblond and Chapman 2002). The second most abundant sterol was 24-methylcholesta-5-en-3β-ol, which had an abundance on par with cholesterol, while the third most abundant sterol was dinosterol at approximately one-third the abundance of cholesterol. Dinosterol is a major sterol in dinoflagellates and serves as an important biomarker for marine organisms (Amo et al. 2010). The other sterols observed in *G. carolinianus* were all more minor, averaging less than 10% relative percentage abundance. Thus, as the three most abundant sterols, the presence of cholesterol, 24-methylcholesta-5-en-3β-ol, and dinosterol are compared below to phylogenetically related armored dinoflagellates. The abundance of cholesterol and dinosterol in *G. carolinianus* would appear to place it within Cluster 5 in the Leblond et al. (2010) classification of dinoflagellates according to sterol composition, where these two sterols are listed as sterols IIa and VIIk. However, the predominance of 24-methylcholesta-5-en-3β-ol (sterol IIc) is quite rare within this cluster (and in other dinoflagellate sterol clusters) as described below.

Phylogenetic data reveal that *Alexandrium* species, *Coolia* species and *Pyrodinium bahamanse* are among the closest relatives to *Gambierdiscus* (Gómez et al. 2015). In a study by Leblond and Chapman (2002), *Alexandrium andersonii* Balech, *Alexandrium minutum* Halim and *Alexandrium tamarense* (Lebour) Balech were found to be rich in both free cholesterol (61% to 81% of free sterols) and esters of cholesterol (80% to 83% of sterol esters). *A. minutum* and *A. tamarense* also contained 27% and 34% dinosterol, respectively, in their free sterol fraction. Ma et al. (2011) worked with 2 clones of *Alexandrium tamarense* (named Alex2 and Alex5)

and revealed that Alex2 was comprised of cholesterol (73%), dinosterol (26%) and β-sitosterol (24*R*-ethylcholest-5-en-3β-ol, 1%), while Alex5 was comprised of cholesterol (55.6%), dinosterol (41.7%), β-sitosterol (2%) and stigmastanol (24*R*-ethyl-5α-cholest-22-en-3β-ol, 0.1%). Interestingly, two isolates of *Alexandrium tamarense* were examined by Piretti et al. (1997) and were found to consist almost entirely of dinosterol, thus indicating a possible spectrum of sterols produced within this genus as this differs from the Leblond and Chapman (2002) and Ma et al. (2011) studies, assuming that the isolates were identified correctly. Notably, none of the examined isolates contained 24-methylcholesta-5-en-3β-ol, one of the major sterols of *G. carolinianus*. As described above, dinoflagellates which produce an abundance of cholesterol and dinosterol are found in Cluster 5 in the Leblond et al. (2010) classification, whereas dinoflagellates, such as those examined by Piretti et al. (1997), which produce only an abundance of dinosterol are found in Cluster 6.

Furthermore, Ellegaard et al. (2018) established a close phylogenetic relationship between some *Alexandrium* species and *Gonyaulax* species. In the Alam et al. (1979) paper, the sterol compositions of five *Gonyaulax* species, *G. acatenella* Whedon & Kofoid, *G. catenella* Whedon & Kofoid, *G. washingtonesis* (=*Alexandrium catenella* (Whedon & Kofoid) Balech), *G. tamaresis* (=*A. tamarense*) and *G. polyedra* F.Stein, were reported. They were comprised majorly of cholesterol (ranging from 42% to 62%) and dinosterol (ranging from 33% to 57%) to indicate an overlap with *G. carolinianus*. Piretti et al. (1997) also examined *G. polyedra* and found its sterols to be dominated by dinosterol at almost 50%, with 4α-methyl-24-ethylcholestan-3β-ol (26.8%), 4,24-dimethylcholest-22-en-3β-ol (18.6%), and 4-methylcholestan-3β-ol (7.2%) as the remaining sterols. Along with the other species mentioned above, taxa with an abundance of cholesterol and dinosterol fall within Cluster 5, and those with an abundance of dinosterol with a paucity of cholesterol fall within Cluster 6. Alam et al.

(1979) did not observe the presence of 24-methylcholesta-5-en-3β-ol, again underscoring this interesting trait of *G. carolinianus*.

In *Coolia* sp., cholesterol accounted for 66% of its free sterols and made up 73% of its sterol esters (Leblond and Chapman 2002). Other free sterols found included dinosterol (21%) and 4α,23,24-trimethyl-5α-cholest-24(28)-en-3β-ol (6%) (Leblond and Chapman 2002). Cholesterol was also found to make up 89% of *Coolia monotis* Meunier free sterols, and 78% of its derived sterol. Interestingly, 24-methylcholesta-5-en-3β-ol was found in trace in *C. monotis* free sterols, while it made up 3% of its sterol esters. Other free sterols present were dinosterol (9%) and 4α,23,24-trimethyl-5α-cholest-24(28)-en-3β-ol (2%) (Leblond and Chapman 2002). These taxa were found within Cluster 5 in the Leblond et al. (2010) classification.

Two isolates of *P. bahamanse* were found to contain cholesterol (>70%) in abundance. Other sterols found were dinosterol (approx. 14%) and 4α-methylgorgostanol (22,23-methylene-4α,23,24-trimethyl-5α-cholestan-3β-ol) (approx. 12%) (Houle et al. 2019). No 24-methylcholesta-5-en-3β-ol was observed in *P. bahamense*, which would fall within Cluster 5 in the Leblond et al. (2010) classification.

In *Pyrocystis* species, *P. lunula* was comprised of cholesterol as its major sterol, while dinosterol was a major sterol in the two strains of *Pyrocystis fusiformis* C.W.Thomson – NOAA (33%) and Scripps (59%). The sterols of *Pyrocystis noctiluca* Murray ex Haeckel strains were comprised of dinosterol (approx. 80%) as their major sterol and cholesterol in trace. Also, *P. lunula* and *P. fusiformis* both possessed 4,24-dimethyl-5α-cholestan-3β-ol, with amounts of 24-methylcholesta-5-en-3β-ol typically less than 5% (Dahmen & Leblond, 2011). *P. lunula* also contained 24-methylcholesta-5,22E-dien-3β-ol and 24-methyl-5α-cholestan-3β-ol (which are present in *G. carolinianus*) (Leblond and Chapman 2002).

In earlier research by Kokke et al. (1982), major sterols of *P. lunula* documented include 4-cholesten-3-one (7.7%), 4α,24*S*-dimethyl-5α-cholestan-3β-ol (29.4%), cholesterol (27%), 5α-cholestan-3β-ol (7.5%) and 24-methylcholesta-5-en-3β-ol (2.2%). It is worth noting that dinosterol was not among the sterols of *P. lunula* recorded in Kokke et al. (1982).

Outside of the closely related armored dinoflagellates described above, 24-methylcholesta-5-en-3β-ol has also been observed rarely, for example, as a minor sterol of the armored dinoflagellate *Fragilidium* sp. (Leblond and Chapman 2002; Mansour et al. 1999). It has also been observed to have a moderate relative percentage abundance (approximately 2-15%) in species of *Heterocapsa* (Alam et al. 1984), and has been observed as a major sterol in the "dinotom" *Kryptoperidinium* (*Peridinium*) *foliaceum* (F.Stein) Lindemann and *Symbiodinium microadriaticum* LaJeunesse, at relative percentage abundances of approximately 30% (Leblond and Chapman 2002).

To conclude, cholesterol and dinosterol are two of the most abundant sterols in *G. carolinianus*, and this is a trait shared with armored dinoflagellates that are related to *G. carolinianus* and which are found in Cluster 5, thus reinforcing via sterol chemotaxonomy their phylogenetic closeness and providing a shared set of general biomarker sterols. However, none of the other armored dinoflagellates have been observed to produce significant amounts of 24-methylcholesta-5-en-3β-ol, which could potentially serve as a *G. carolinianus* biomarker in the environment. Although hypotheses have been made to determine some of the steps in dinoflagellate sterol biosynthesis, for example in Giner et al. (2016), the complete biosynthetic pathways that lead to the diversity of their sterols are largely unknown. However, it can be inferred, particularly in the case of cholesterol-containing dinoflagellates, that their biosynthesis involves many of the steps common to the biosynthesis of sterols in better-studied eukaryotes [summarized by Nes (2011)].

G. carolinianus largely appears to be a benthic species on the surface of macrophytes rather than a bloom-former (Tester et al. 2013). Future research should focus on two questions. First, does the sterol profile, specifically the high abundance of 24-methylcholesta-5-en-3β-ol, of field-collected cells match what we have observed in pure culture, if sufficient biomass can be separated and harvested from macrophyte surfaces in a manner similar to Richlen et al. (2024), with steps taken to limit the contribution of macrophyte biomass to the sterol profile of collected material? Second, to what extent does the sterol composition of *G. carolinianus* overlap with other *Gambierdiscus* species? There is nothing in the literature to date. Thus, to this end, we attempted to grow other species of *Gambierdiscus*, including *G. belizeanus* M.A.Faust (ARC 397), *G. caribaeus* Vandersea, Litaker, M.A.Faust, Kibler, W.C.Holland & P.A.Tester (ARC 398), *G. carpenteri* Kibler, Litaker, M.A.Faust, W.C.Holland, Vandersea & P.A.Tester (ARC 399), and *G. pacificus* Chinain & M.A.Faust (ARC 349), also obtained from ARC, but were unsuccessful. At this point, the abundance of 24-methylcholesta-5-en-3β-ol appears to be a chemotaxonomic uniqueness of *G. carolinianus* within those closely related armored dinoflagellates examined to date.

Author contributions

Tawakalit J. Busari: Investigation, Writing – original draft. Braedyn E. Hollingsworth: Investigation. Isaac E. Rippy: Investigation Seth I. Spurlock: investigation. Jeffrey D. Leblond: Investigation, Writing – review & editing.

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