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SHORT COMMUNICATION

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First examination of sterols in the marine dinoflagellate genus *Vulcanodinium*

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Abstract

Vulcanodinium is an ecologically relevant dinoflagellate genus due to its production of neurotoxins known as pinnatoxins. We present here the first examination of the sterols of a *Vulcanodinium rugosum* isolate. Sterols are ringed lipids that assist in maintaining rigidity of cellular membranes, and the Dinophyceae are well-studied for their ability to produce a diverse array of sterols, many of which have chemotaxonomic utility. We have determined that *V. rugosum* produces a set of major sterols, namely cholesterol, dinosterol, 4α,24-dimethyl-5α-cholest-22E-en-3β-ol, and 4α,24-dimethyl-5α-cholestan-3β-ol, common to the Dinophyceae. However, this displayed marked differences from those studied members of the genera *Scrippsiella* and *Peridinium*, the closest phylogenetic relatives. Included in these differences is production by *V. rugosum* of a much lower percentage of dinostanol, a saturated form of dinosterol.

KEYWORDS
dinoflagellate, Dinophyceae, lipid, sterol, *Vulcanodinium*

INTRODUCTION

Vulcanodinium rugosum E. Nézan & N. Chomérat is an armored marine dinoflagellate initially discovered off the French Mediterranean coast (Nézan & Chomérat, 2011). *Vulcanodinium rugosum* is considered to be the producer of the pinnatoxin found, for example, in the filter-feeding fan mussel species *Pinna attenuata* (Rhodes et al., 2011). As such, *V. rugosum* has been identified as a causative organism of many cases of shellfish poisoning in regions such as Japan, Australia, New Zealand, and China (Garrett et al., 2014). Pinnatoxins are macrocyclic compounds that inhibit neural and muscular nicotinic acetylcholine receptors, which can lead to an onset of neurological symptoms and, in cases of high dosages and long-term ingestion, death (Geiger et al., 2013). To the best of our knowledge, literature suggests the *Vulcanodinium* genus is the sole producer of pinnatoxins, where the toxins are regularly documented within bivalve populations and are found in low amounts in both sediment and water samples taken from several marine locations around the globe (Lamas et al., 2019).

Vulcanodinium rugosum is the only named species within the genus *Vulcanodinium*. The closest relatives, as defined by large ribosomal subunit (LSU) rRNA and internal transcribed spacer (ITS) sequence data, are members of the Peridiniales (Nézan & Chomérat, 2011), though the hypotheca and cingular plates of *Vulcanodinium* suggest possible relation to the order Gonyaulacales (Zeng et al., 2012). Specifically, *V. rugosum* is related to the peridinioid species *Ensiculifera* aff. *Loeblichii* E.R. Cox & H.J. Arnott, *Ensiculifera imariensis* S. Kobayashi & K. Matsuoka, *Pentapharsodinium tyrrhenicum* (Balech) Montresor, Zingone & Marino ex Head, *Peridinium aciculiferum* Lemmermann, *Peridinium pseudolaeve* M. Lefèvre, and *Scrippsiella trochoidea* (F. Stein) A.R. Loeblich III (Rhodes

et al., 2011). The close relationship of *V. rugosum* to these species is emphasized in a phylogeny based on internal transcribed spacer regions (ITS1 and ITS2) and the gene for 5.8S rRNA (Figure S1, described in more detail below). Note that *Scrippsiella* is a member of the Thoracosphaerales, while the other genera listed are members of the Peridiniales (per the algal taxonomy database AlgaeBase.org).

Dinoflagellates have been studied for decades as producers of dozens of sterols, many rarely found in other classes of algae (Volkman, 1986, 2003; Volkman et al., 1998). A sterol is a ringed lipid that serves to add rigidity to the phospholipid bilayer (Dufourc, 2008). Some dinoflagellate sterols, such as C_{30} dinosterol (4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol) and C_{27} cholesterol (cholest-5-en-3β-ol), are together indicative, for example, of phylogenetically diverse genera such as *Alexandrium* (Leblond & Chapman, 2002; Piretti et al., 1997) and certain species of *Prorocentrum* (Leblond & Chapman, 2002; Volkman et al., 1999; dinoflagellate phylogeny reviewed in Janouškovec et al., 2017). Conversely, dinosterol in the (relative) absence of cholesterol is found as a major sterol (often with other 4α-methyl-substituted sterols as major sterols), for example, in *Heterocapsa* (Alam et al., 1984) and other species of *Prorocentrum* (Volkman et al., 1999). Note that in many dinoflagellates, there are also other sterols, some with and some without the 4α-methyl group found in dinosterol (Leblond et al., 2010 and references therein). 4α-Methyl-substituted sterols such as dinosterol are often considered to be chemotaxonomically useful representations of the class Dinophyceae as a whole.

A framework for classifying dinoflagellates according to sterol composition is presented in Leblond et al. (2010); within this framework, some of the chief determinants for assigning a dinoflagellate to a sterol "cluster" are the presence of cholesterol and/or dinosterol as the major sterol, or the presence of more distinctive 4α-methyl-substituted sterols as in members of the Kareniaceae (Leblond et al., 2010 and references therein). As seen with the members of the genus *Prorocentrum*, a single genus within an order (i.e. Prorocentrales) can be present in two different clusters depending on the major sterols present, although in general sterol composition tends to reflect groupings as determined by rRNA sequences (Leblond et al., 2010).

One of the continuing goals of our laboratory is to elucidate the sterol composition and provide chemotaxonomic inference of recently identified dinoflagellates once they become available by culture collections. To our knowledge, the genus *Vulcanodinium* has not until recently been publicly available from a culture collection; thus, we are presenting the sterol composition of *V. rugosum* as the first reported sterol composition of the genus *Vulcanodinium*.

MATERIALS AND METHODS

Culturing

Vulcanodinium rugosum UNCW-ARC 382 isolated from ship ballast water in Florida was acquired from the Algal Resources Collection (www.algalresourcescollection.com) and grown in triplicate in L1 medium (Guillard & Hargraves, 1993) at 20℃, a light/dark cycle of 14/10 h, and a light intensity of approximately 50 µM photons/m^2/s using a combination of cool white fluorescent and LED bulbs. Cells were harvested via filtration onto Whatman 934-AH glass microfiber filters (GE Healthcare) during the exponential phase of growth when cells were at a concentration of approximately 10^4 cells/ml. Filters were preserved at −80℃ until lipid extraction.

Phylogenetic confirmation of *Vulcanodinium rugosum* UNCW-ARC 382 identification

In order to assure that the UNCW-ARC 382 isolate, which was originally identified as *Vulcanodinium* sp., is indeed *V. rugosum*, phylogenetic characterization was performed according to the following methodology: 50 ml of culture was centrifuged, and the obtained pellet frozen in liquid nitrogen. DNA was then extracted following the CTAB protocol (Lebret et al., 2012). The internal transcribed spacers ITS1 and ITS2 and the 5.8S rDNA were amplified using primers 329f (5′-GTG AAC CTG CRG AAG GAT CA-3′) and D1R-R (5′-TAT GCT TAA ATT CAG CGG GT-3′; Arsenieff et al., 2019). The 15 µl PCR amplification mix contained 1 µl of the DNA extract, 330 µM of each deoxynucleoside triphosphate (dNTP), 2.5 mM of $MgCl_2$, 1.25 U of GoTaq® DNA polymerase (Promega Corporation), 0.17 µM of both primers, and 1× of buffer (Promega Corporation). Thermocycler conditions followed Arsenieff et al. (2019). Fresh PCR product was cloned using the TOPO TA cloning with the pCR4-TOPO TA vector (Invitrogen) following the manufacturer's instructions. The PCR product of one positive clone was purified (ExoSAP-IT®; Affymetrix) using the Big Dye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems) and sequenced using the primers in both forward and reverse directions using primers 329f and D1R-R. The online software MAFFT 7 (https://mafft.cbrc.jp/alignment/server) was used to align the *V. rugosum* obtained in this study (GenBank accession no. MZ189363), along with ITS sequences of *V. rugosum* and other dinoflagellates (HQ845329, EU728696, MT895109, KY404227) obtained

from GenBank. The best nucleotide substitution model was determined using MEGA7 (Kumar et al., 2016), and the Kimura-2-parameter model (Kimura, 1980) was selected with invariant sites (K2+I) and partial deletion of gaps. Maximum likelihood was measured using MEGA7, and the robustness of the inferred topology was supported by bootstrap resampling (1000 replicates).

Lipid processing and sterol analysis

Total lipids were extracted and fractionated according to polarity using the procedure described by Leblond and Chapman (2000). The sterol ester and free sterol fractions were saponified and derivatized to form trimethylsilyl (TMS)-ether derivatives of sterols according to published procedures (Leblond & Chapman, 2002). The derivatives were analyzed via gas chromatography/mass spectrometry (GC/MS) with a Thermo TSQ Quantum GC/MS and a Restek Rxi-5Sil MS column (30 m × 0.25 μm film thickness, Restek Corp.) in positive-ion electron impact (EI) mode using the instrument conditions described by Houle et al. (2019). Data files (i.e. chromatograms and associated mass spectra) were processed using Thermo Xcalibur ver. 2.1. Relative retention times (RRT) to cholesterol (standard purchased from Alfa Aesar) were calculated using retention times (RT) according to the methodology of Jones et al. (1994). Compound identifications were made by comparing to the RRT values and published mass spectra data of Jones et al. (1994), to the studies listed in the Figure S2 legend, and to additional published works referenced in Leblond and Chapman (2002) and Leblond et al. (2010).

Chemotaxonomic analysis of sterol data

A clustergram (Figure S2) of the chemotaxonomic relationship of *V. rugosum* to other dinoflagellates was created using the Primer-e software package (Quest Research Limited). This clustergram is based on a Bray–Curtis similarity resemblance matrix of untransformed relative percentage sterol composition data from the published works listed in the Figure S2 legend. In order to express the driving factors behind the clustering patterns, a shade plot (i.e. heat map, Figure S3) was also generated using Primer-e with the clustergram of Figure S2 positioned along the *Y*-axis and the relative percentage data from the studies listed in the Figure S2 legend displayed using the color scale.

RESULTS AND DISCUSSION

The phylogenetic analysis of the transcribed spacers ITS1 and ITS2 and the 5.8S rDNA region by ML revealed that *V. rugosum* UNCW-ARC 382 clustered together with *V. rugosum* sequences from Cuba, Japan, and Qatar (Figure S1).

A total of fifteen sterols and two steroidal ketones were observed in *V. rugosum* (Table 1). The vast majority (approx. 98%) of the sterols was found within the free sterol fraction, while a much smaller amount of sterols and two steroidal ketones were found within the sterol ester fraction.

Within the free sterol fraction, the major sterols were (in order of most abundant to least abundant) C_{29} 4α,24-dimethyl-5α-cholest-22E-en-3β-ol, dinosterol, cholesterol, and C_{29} 4α,24-dimethyl-5α-cholestan-3β-ol (Table 1). These four sterols were also among the most abundant sterols in the sterol ester fraction along with an unidentified C_{28} sterol. Also found within the sterol ester fraction were two steroidal ketones, 4α-methyl-5-en-3-one and 4α,24-dimethyl-5α-cholestan-3-one. A number of less abundant minor sterols were also observed, many of which possessed a 4α-methyl group, in both fractions.

Of the dinoflagellates shown to be closely related to *V. rugosum* per Rhodes et al. (2011), sterol composition data exist for *P. aciculiferum*, *S. trochoidea*, and *Scrippsiella* sp. Thus, the following text will describe similarities and differences between their sterols and those of *V. rugosum*. Figures S2 and S3 coincide with this text to provide a representation of the chemotaxonomic relationship of *V. rugosum* to the closely related dinoflagellates described below, along with a selection of other more distantly related dinoflagellates. The dinoflagellates in Figures S2 and S3 represent examples of Clusters 5 and 6 as originally described in Leblond et al. (2010), where it was shown that photosynthetic and heterotrophic dinoflagellates form six clusters according to sterol composition. In brief, Cluster 5 dinoflagellates are generally enriched in cholesterol and dinosterol (and other sterols with the same ring systems), whereas Cluster 6 dinoflagellates are generally enriched in dinosterol (and other sterols with the same ring system). At the time of the Leblond et al. (2010) study, there were approximately forty dinoflagellates with approximately ten to fifteen genera represented in each of these two clusters—if the analysis were repeated now, there would undoubtedly be more to account for more recent published works.

TABLE 1 Sterol composition of *Vulcanodinium rugosum* UNCW-ARC 382

Carbon #	Suggested structure (common name where available)	Molecular weight[a]	Retention time (min)	RRT[b]	Free sterols	Sterol esters
C_{27}	Cholest-5-en-3β-ol (cholesterol)	458	37.67	1	16.9 ± 2.0	6.6 ± 0.9
C_{27}	5α-Cholestan-3β-ol (cholestanol)	460	37.76	1.03	2.0 ± 0.3	1.5 ± 0.4
Unknown	Unidentified sterol with indeterminate # of carbons and double bonds	Unclear	37.82	1.04		1.1 ± 0.2
C_{27}	Unidentified $C_{27:2}$ sterol	456	38.12	1.12	1.1 ± 0.1	
C_{27}	Unidentified $C_{27:1}$ sterol	458	38.29	1.17	0.2 ± 0.1	
C_{28}	4α-Methyl-5-en-3-one (4-methylcholest-3-one)[c]	400	38.45	1.21		0.8 ± 0.3
C_{28}	4α-Methyl-5α-cholest-7-en-3β-ol (lophenol) or 4-methyl-5α-cholest-8-en-3β-ol	472	38.6	1.25	0.4 ± 0.0	Tr
C_{28}	4α-Methyl-5α-cholestan-3β-ol (4-methylcholestanol)	474	38.8	1.31	1.5 ± 0.3	1.3 ± 0.0
C_{28}	Unidentified $C_{28:0}$ sterol	474	39.02	1.38	0.7 ± 0.2	38.8 ± 16.7
C_{29}	4α,24-Dimethyl-5α-cholest-22E-en-3β-ol	486	39.53	1.51	36.2 ± 1.1	20.9 ± 14.9
C_{29}	Unidentified $C_{29:2}$ sterol	484	39.83	1.59	2.2 ± 0.3	
C_{29}	4α,24-Dimethyl-5α-cholestan-3-one[c]	414	39.83	1.59		5.5 ± 2.4
C_{29}	23,24-Dimethyl-5α-cholest-22E-en-3β-ol	486	39.99	1.63	4.4 ± 0.8	6.5 ± 2.6
C_{30}	Unidentified $C_{30:2}$ sterol	498	40.27	1.71		4.0 ± 1.3
C_{29}	4α,24-Dimethyl-5α-cholestan-3β-ol	488	40.29	1.72	13.3 ± 1.7	5.0 ± 1.8
C_{30}	4α,23,24-Trimethyl-5α-cholest-22E-en-3β-ol (dinosterol)	500	40.57	1.79	19.9 ± 0.7	9.7 ± 3.4
C_{30}	4α,23,24-Trimethyl-5α-cholestan-3β-ol (dinostanol)	502	41.45	2.03	0.7 ± 0.2	0.5 ± 0.5
Percentage of total sterols					98.0 ± 1.3	1.1 ± 1.2

Note: Tr-present at < 0.2%.

[a]Molecular weight as sterol as a TMS derivative or underivatized steroidal ketone.

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

[c]Steroidal ketone not as a TMS derivative.

Scrippsiella trochoidea and *Scrippsiella* sp. CS-295/c were examined by Mansour et al. (1999) and were found to differ modestly from each other. Notably, *Scrippsiella* sp. CS-295/c possessed cholesterol, dinosterol, and C_{30} dinostanol (4α,23,24-trimethyl-5α-cholestan-3β-ol) as the three most abundant sterols at relative percentages of approximately 24%, 31%, and 18%, respectively. In contrast, *S. trochoidea* was found to have a larger number of sterols with cholesterol and dinosterol at much lower relative percentages of approximately 1 and 8%, respectively, while dinostanol was the most abundant sterol at approximately 33%. *S. trochoidea* examined by Harvey et al. (1988) and Leblond and Chapman (2002) were enriched in dinostanol and similarly had little to no cholesterol per the *S. trochoidea* examined by Mansour et al. (1999).

Peridinium aciculiferum and the related *Scrippsiella hangoei* (J. Schiller) J. Larsen (Logares et al., 2007) were examined by Leblond et al. (2006) and found to have dinostanol as the dominant sterol. In contrast to the *Scrippsiella* isolates described just above, both also had high levels of C_{30} 4α,23,24-trimethyl-5α-cholest-7-en-3β-ol and C_{27} 5α-cholestan-3β-ol (cholestanol) as major sterols. *P. aciculiferum* and *S. hangoei* lacked cholesterol in a manner similar to the little to no cholesterol observed in *S. trochoidea* by Harvey et al. (1988) and Leblond and Chapman (2002).

To summarize the most significant similarities and differences between *V. rugosum* and *S. trochoidea*, *Scrippsiella* sp. CS-295/c, *P. aciculiferum*, and *S. hangoei*, the following points are noted with regard to free sterols (which represented the overwhelming majority of the sterols found in *V. rugosum*):

- *V. rugosum* possessed 4α,24-dimethyl-5α-cholest-22E-en-3β-ol as the most abundant sterol. This was a minor sterol in *S. trochoidea* (Harvey et al., 1988; Leblond & Chapman, 2002; Mansour et al., 1999) and *Scrippsiella* sp. CS-295/c (Mansour et al., 1999) and was absent in *P. aciculiferum* and *S. hangoei* (Leblond et al., 2006).

- The relative percentage of 4α,24-dimethyl-5α-cholestan-3β-ol in *V. rugosum* was slightly higher than the relative percentage of this sterol in *S. trochoidea* (Harvey et al., 1988; Leblond & Chapman, 2002; Mansour et al., 1999), *Scrippsiella* sp. CS-295/c (Mansour et al., 1999), and *P. aciculiferum* and *S. hangoei* (Leblond et al., 2006).
- Dinosterol was the second most abundant sterol in *V. rugosum* with a relative percentage similar to *S. trochoidea* (Harvey et al., 1988; Leblond & Chapman, 2002; Mansour et al., 1999), *Scrippsiella* sp. CS-295/c (Mansour et al., 1999), and *P. aciculiferum* (Leblond et al., 2006).
- *V. rugosum* possessed much less dinostanol (< 1%) than *S. trochoidea* (Harvey et al., 1988; Leblond & Chapman, 2002; Mansour et al., 1999), *Scrippsiella* sp. CS-295/c (Mansour et al., 1999), and *P. aciculiferum* (Leblond et al., 2006).
- *V. rugosum* possessed cholesterol as a major sterol at approximately 17%. This is similar to *Scrippsiella* sp. CS-295/c (Mansour et al., 1999) in terms of relative percentage, but more than *S. trochoidea* (Harvey et al., 1988; Leblond & Chapman, 2002; Mansour et al., 1999) and *P. aciculiferum* and *S. hangoei* (Leblond et al., 2006). As an additional note, the genus *Heterocapsa* is arguably the best studied member of the Peridiniales in terms of sterol composition, and it too has generally been found to possess little to no cholesterol (Alam et al., 1984; Leblond & Chapman, 2002).

At the current time, it is apparent that *V. rugosum* produces within its major sterols cholesterol and dinosterol; this is generally consistent in a qualitative sense with many phylogenetically diverse dinoflagellates (*cf.* Leblond et al., 2010). However, the relative percentage distribution of these two sterols and 4α,24-dimethyl-5α-cholest-22E-en-3β-ol differs from the dinoflagellates currently described as the closest relatives to *Vulcanodinium* per Rhodes et al. (2011). More specifically, using the classification scheme of Leblond et al. (2010), the high relative percentages of cholesterol and dinosterol appear to place *V. rugosum* within Cluster 5, whereas the species described above are firmly placed in Cluster 6 due to their general absence/low amount of cholesterol. It is important to note, however, that this is the first examination of the sterols of the pinnatoxin-producing genus *Vulcanodinium*, and that as more isolates become available for study, expanded examinations of their sterols will determine whether our observations are generally consistent with other isolates from the genus, including *V. rugosum*.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Figure S1. ITS phylogeny (ITS-1/5.8S/ITS-2) showing the relationship between Vulcanodinium rugosum UNCW-ARC 382 (in bold) and other V. rugosum strains.
Figure S2. Bray-Curtis similarity clustergram of Vulcanodinium rugosum UNCW-ARC 382 and other dinoflagellates as based on relative percentages of sterols.
Figure S3. Bray-Curtis similarity clustergram of of Vulcanodinium rugosum UNCW-ARC 382 and other dinoflagellates as based on relative percentages of sterols.

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