The lipid composition of the marine dinoflagellate *Zooxanthella nutricula*, a polycystine radiolarian symbiont

by
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Abstract

Dinoflagellates are important primary producers in their marine and freshwater environments, and they form symbiotic relationships with larger marine organisms. Previous research has explored symbiotic relationships such as cnidarian symbioses with *Symbiodinium* dinoflagellates. However, little research addresses the symbiotic relationship between radiolarians and their symbionts, such as the marine dinoflagellate *Zooxanthella nutricula*. An important aspect of dinoflagellate symbiosis involves the exchange of metabolites such as the transfer of lipids from the symbiont to the host. This research aimed to identify the lipid content, particularly that of sterols and fatty acid-containing galactolipids, of *Z. nutricula*. The major sterol identified was 22-dehydrocholesterol, which does not tend to be a dominant sterol among dinoflagellates. The major galactolipid was 18:5/18:5 MGDG, and all galactolipids identified were C_{18}/C_{18} forms of MGDG and DGDG. These results along with future research about radiolarian lipids should elucidate the role of sterols and fatty acids in dinoflagellate-radiolarian symbiosis.

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Introduction

Dinoflagellate Symbioses with Larger Organisms

Marine and freshwater dinoflagellates are phytoplankton that play an important role in aqueous environments due to their ability to fix carbon and to provide chemical energy that continues up the food chain (Hackett et al., 2004; Spector, 1984). About half of all documented dinoflagellates are capable of photosynthesis and have photosynthetic pigments, such as the carotenoid peridinin, that are responsible for the cell's overall pigmentation (Dodge, 1984; Gaines & Elbrächter, 1987). In contrast, the other half of dinoflagellates are heterotrophic and are not capable of photosynthesis due to a lack of the necessary photosynthetic pigments and chloroplasts (Hackett et al., 2004). As a result, these heterotrophic cells require food and energy from other organisms or acquire nutrients from the environment (Sleigh, 2000).

Some dinoflagellates are zooxanthellae and engage in symbiotic relationships with many different marine organisms such as protozoan radiolarians and foraminifera, and cnidarian animals such as anemones, jellyfish, and corals (Gast & Caron, 2001; Gordon & Leggat, 2010). This interaction between dinoflagellates and host organisms is significant for the environment because dinoflagellates, as important primary producers, fix carbon dioxide produced by the host (Yellowlees et al., 2008). Symbiosis is also beneficial for the host because as dinoflagellates recycle carbon, they provide photosynthetic products such as glucose, lipids, proteins and amino acids, and other metabolites for the host to be used for different biological processes (Hackett et al., 2004; Trench, 1971; Yellowlees et al., 2008). In return, the host provides inorganic nutrients such as nitrogen as ammonium and phosphorus as phosphate for the symbiont so that the

cyclical transfer of metabolites continues (Muscatine & Porter, 1977; Yellowlees et al., 2008). However, under conditions of extreme heat and light, this symbiotic relationship can be impaired and result in the expulsion of symbiont dinoflagellates from the host, which can harm both organisms (Villar et al., 2018). The termination of symbiont-coral interactions can result in coral bleaching, which causes concern about rising water temperatures because bleaching can cause the coral to be more susceptible to diseases, inhibit reproduction, and cause coral death (Baird & Marshall, 2002; Miller et al., 2009).

Different classes of lipids are important for processes within dinoflagellates and hosts (Gordon & Leggat, 2010). Triglycerides provide energy storage for cells, glycolipids in the photosynthetic symbiont constitute chloroplast membranes and help regulate photosystems, phospholipids and betaine lipids form cell membranes, and sterols provide structural support for cell membranes (Kumari et al., 2013). Symbiotic relationships are important because symbiotic dinoflagellates, particularly the genus *Symbiodinium*, can synthesize and transfer sterols, such as cholesterol (cholest-5-en-3β-ol) and gorgosterol (22,23-methylene-23,24-dimethyl-cholest-5-en-3β-ol), that their host is unable to produce (Goad, 1981; Hambleton et al., 2019; Withers et al., 1982). Cholesterol is an important component of cell membranes, and while gorgosterol is mainly produced and transferred by dinoflagellates in symbiosis, its function in the host is unknown (de Oliveira Andrade, 2016; Rampen et al., 2009). The host benefits from these sterols because they can provide stability for cell membranes, and because cnidarian hosts are unable to synthesize their own sterols, they must rely on those that are produced by symbionts or acquired by consuming prey (Baumgarten et al., 2015; Hambleton et al., 2019).

Radiolarians, which are marine protists known to host dinoflagellates, typically live in nutrient-lacking, open ocean environments and can live as a single organism or as a cluster of cells (Anderson, 2001). Dinoflagellates and radiolarians form an important symbiotic relationship in oligotrophic environments since the symbiotic dinoflagellates presumably provide nutrients for the host (Anderson, 2001; Liu et al., 2019; Trench, 1987). In return, the host provides ammonium to the symbiont which is significant because oligotrophic waters tend to be nitrogen-deficient (Liu et al., 2019). Radiolarians are also able to consume symbiotic dinoflagellates as food, as evidenced by the radiolarian *Collozoum inerme*, found to have ingested dinoflagellates that resembled those of the genus *Aureodinium* which presently includes only the species *A. pigmentosum* Dodge (Anderson, 1976; Dodge, 1967; Guiry & Guiry, 2020). Whether by transferring metabolites or being digested, dinoflagellates play a crucial role in radiolarian nutrition. (Suzuki & Not, 2015).

In addition to hosting dinoflagellates, radiolarians are also important in the field of paleoclimatology (Haq & Boersma, 1998). Different characteristics and patterns of preserved radiolarians in ocean sediments and living radiolarians within the water column reflect changes in climate conditions such as water temperature (Haq & Boersma, 1998). For example, there is a possible association between sea surface temperature and the frequency that polycystine radiolarians assemble in which multiple species congregate together (Suzuki & Not, 2015). Polycystine radiolarians are radiolarians that produce a silica (silicon dioxide) skeleton and include only the orders Spumellaria, Nassellaria, and Collodaria while the two remaining orders of Acantharia and Taxopodia are not considered polycystine (De Wever et al., 2001; Suzuki & Not, 2015). Since different

species of radiolarians prefer different environments, the species present in the assemblages can provide insight about climatic and environmental conditions (Lampitt et al., 2009). While radiolarians strongly impact organic carbon cycles with photosynthetic symbionts, they also impact inorganic cycles in the ocean (Suzuki & Not, 2015). Radiolarians in the order Acantharia use strontium and barium to build strontium sulfate skeletons, and those in the orders Spumellaria, Nassellaria, and Collodaria use dissolved silica to build opaline silica skeletons (Canfield et al., 2005; Suzuki & Not, 2015). Radiolarian remains are thus important contributors to silica found in ocean sediments (Canfield et al., 2005; Suzuki & Not, 2015). Radiolarians contribute to inorganic cycles by building skeletons, and together, dinoflagellates and radiolarians contribute to organic cycles (e.g. carbon) through dinoflagellate photosynthesis and to inorganic cycles (e.g. nitrogen and phosphorus) through the transfer of nutrients.

Radiolarians within the orders Collodaria, Spumellaria, and Nassellaria can live symbiotically with the dinoflagellate *Zooxanthella nutricula* K.Brandt (Brandt, 1881/2016; Probert et al., 2014; Yuasa et al., 2016). *Z. nutricula* was first identified in the late 1800s by Karl Brandt; however, he provided vague descriptions of the dinoflagellate which caused confusion about different species and their relation to the genus *Zooxanthella* (Yuasa et al., 2016). As a result of the confusion, *Z. nutricula* was also given the name of *Brandtodinium nutricula* (K.Brandt) Probert & Siano, but *Z. nutricula* has since been regarded as the valid name of this particular species of dinoflagellates (Krueger, 2016; Yuasa et al., 2016). However, it is important to note that while the term zooxanthellae describes dinoflagellates that are capable of symbiosis with other marine

organisms, the genus *Zooxanthella* does not encompass all zooxanthellae dinoflagellates (Krueger, 2016).

In addition to *Z. nutricula* (peridinioid), radiolarians can engage in symbiosis with the dinoflagellates *Amphidinium* sp. (amphidinioid) and the recently discovered *Gymnoxanthella radiolariae* T.Yuasa & T.Horiguchi (gymnodinioid; Taylor, 1974; Yuasa et al., 2016). The terms peridinioid, amphidinioid, and gymnodinioid describe dinoflagellates within phylogenetic groups as based on cell morphologies and gene sequences (Medlin & Cembella, 2013). Therefore, radiolarians are capable of forming symbiotic relationships with phylogenetically different dinoflagellates (Yuasa et al., 2016). Further discussion of the phylogeny of dinoflagellates symbiotic with radiolarians is covered later.

Symbiotic dinoflagellates, mostly in the genus *Symbiodinium*, are important for marine invertebrates such as corals and anemones (i.e. cnidarians) because they transfer lipids, especially sterols, in addition to nutrients to the cnidarian host (Goad, 1981; Gordon & Leggat, 2010). While both *Z. nutricula* (Peridiniales) and *Symbiodinium* (Suessiales) engage in symbiotic relationships, they are not closely related phylogenetically, and the host organisms, radiolarians and anthozoans, respectively, are different (Probert et al., 2014). *Symbiodinium* dinoflagellates within the order Suessiales comprise an important phylogenetic group because they tend to be symbionts, so they are typically classified as zooxanthellae (Medlin & Cembella, 2013). However, other dinoflagellates not within the Suessiales are capable of forming symbiotic relationships with marine organisms, as evidenced by *Z. nutricula* which is within the order Peridiniales (Decelle et al., 2012; Probert et al., 2014). Groups of closely related

dinoflagellates tend to be symbionts of the same host organism (Gast & Caron, 2001). Therefore, the different hosts of *Z. nutricula* and *Symbiodinium* could reflect phylogenetic differences between the dinoflagellates since *Z. nutricula* forms symbiotic relationships with radiolarians, and *Symbiodinium* form symbiotic relationships with anthozoans within the phylum Cnidaria and with protozoan foraminifera (Gast & Caron, 2001; Gottschling & McLean, 2013). Within foraminifera and cnidarian symbiotic relationships with *Symbiodinium* species, metabolite exchanges are similar in that lipids and glycerol are transferred to the host, and nutrients such as nitrogen and phosphorus are transferred to the symbiont (Hallock, 2007; Lee, 1995).

Radiolarians and foraminifera are both classified within the eukaryotic supergroup Rhizaria and are further grouped together within the Retaria clade (Burki et al., 2010; Moreira et al., 2007; Sierra et al., 2013). Phylogenetic trees support the placement of foraminifera as a sister group to either the radiolarian order Acantharia or to polycystine radiolarians (Burki et al., 2010; Sierra et al., 2013). Therefore, radiolarians and foraminifera are closely related even though they engage in symbiosis with phylogenetically different dinoflagellates. While research about radiolarian symbiosis is not as extensive as cnidarian and foraminifera symbioses in terms of metabolite exchanges and interactions between the symbiont and host, it is believed that metabolite exchanges similar to those in cnidarian and foraminifera symbiotic interactions occur during radiolarian-dinoflagellate symbiosis (Liu et al., 2019). Important metabolite exchanges involved in dinoflagellate-radiolarian symbiosis include the transfer of ammonium to the symbiont and the possible transfer of amino acids to the host (Liu et al., 2019).

In addition to forming symbiotic relationships and contributing to carbon cycles, the production of toxins is a characteristic of some dinoflagellates, recognizing that there are many different types of dinoflagellate toxins and that these differences can reflect phylogenetic differences between the dinoflagellates that produce them (Medlin & Cebella, 2013). As a result, the presence of toxins does not necessarily indicate a phylogenetic relatedness between dinoflagellates since toxic dinoflagellates can be found in different phylogenetic groups (Medlin & Cebella, 2013).

Most dinoflagellate toxins, such as the collection of brevetoxins produced by *Karenia brevis* (C.C.Davis) Gert Hansen & Moestrup are neurotoxins that are often associated with harmful algal blooms (HABs) caused by free-swimming planktonic and benthic dinoflagellates not in symbiosis with a larger organism (Hackett et al., 2004). These neurotoxins can negatively affect a variety of higher organisms including humans, marine mammals, and fish (Hackett et al., 2004; Medlin & Cebella, 2013). Whether or not toxin production is a general characteristic of zooxanthellae, and if so, the effect on the host organism, is an open question. The predominant dinoflagellates responsible for HABs are not of the genus *Symbiodinium* (Hackett et al., 2004). However, *Symbiodinium* dinoflagellates have been found to produce zooxanthellatoxins (ZTs) such as ZT-A and ZT-B as well as the chemically related symbiodiniolide and zooxanthellamides (Gordon & Leggat, 2010; Kita et al., 2007; Nakamura et at., 1993; Onodera et al., 2003). These toxins functionally resemble other vasoconstrictive toxins such as maitotoxin and palytoxin, but they are structurally different (Nakamura et al., 1993). ZTs are harmful because they open calcium ion channels, and increased calcium ion concentrations induce their vasoconstrictive properties which can harm tissues (Nakamura et al., 1995; Moriya

et al., 1998). The reason why *Symbiodinium* produces toxins is not well understood, but they are believed to be part of the complex symbiosis between zooxanthellae and the host organism since ZTs function by opening calcium ion channels in the host (McConnaughey, 2012). Toxins could be a way for zooxanthellae to regulate the delicate balance of biological pathways such as calcification and nutrient acquisition in a way that benefits both the host and symbiont (McConnaughey, 2012). Typical *Symbiodinium* hosts such as coral, foraminifera, and sponges are calcareous, so they undergo calcification to build their skeleton (McConnaughey, 2012). Products of calcification include the calcium carbonate skeleton as well as carbon dioxide, produced when bicarbonate (HCO_3^-) reacts with protons released during calcification, which zooxanthellae use for photosynthesis (McConnaughey, 2012; Toyofuku et al., 2017). Calcification is also thought to improve nutrient uptake in zooxanthellae (McConnaughey, 2012; McConnaughey & Whelan, 1997). The host organism has sole control over the release of nutrients to the symbiont, so when nutrient levels are low, zooxanthellae toxins could stimulate calcification as a way to acquire nutrients (McConnaughey, 2012; Rands et al., 1993). However, the release of excessive toxins and enhanced nutrient uptake by zooxanthellae could cause an increased abundance of symbionts which could ultimately harm the host through the loss of nutrients (McConnaughey, 2012; Tanaka et al., 2007). Zooxanthellae can leave the host due to lack of nutrients or be expelled by the host due to the production of too many toxins, but the host could be harmed when too many symbionts leave at once (McConnaughey, 2012). Zooxanthellae toxins and the ability of only the host to release nutrients to the symbiont regulate an equilibrium in which enough nutrients need to be released to the symbiont to prevent the release of toxins but not enough to cause

overgrowth of zooxanthellae which can harm the host through nutrient depletion (McConnaughey, 2012; Rands et al., 1993).

The presence of toxins in *Z. nutricula* is unknown, and although it is considered a type of zooxanthellae, it most likely does not produce ZTs due to its distant phylogenetic relatedness to *Symbiodinium* and because its radiolarian host does not have a calcium skeleton (Suzuki & Not, 2015). The genus *Heterocapsa* which is related to *Z. nutricula* (see below) contains dinoflagellates symbiotic with Acantharia which also lack a calcium skeleton (Suzuki & Not, 2015). However, the genus *Heterocapsa* includes, for example, *H. circularisquama* Horiguchi which produces harmful photosensitizing hemolytic toxins, H2-a and H3-b, which are structurally different than ZTs (Miyazaki et al., 2005; Nakamura et al., 1995; Onodera et al., 2003; Sato et al., 2002). The recently discovered species *H. bohaiensis* J.Xiao & Y.Li also produces hemolytic toxins, and further research will clarify the role and mechanism of these toxins (Zhang et al., 2019). Apart from *H. circularisquama* and *H. bohaiensis*, no published research indicates that other *Heterocapsa* species produce toxins.

Dinoflagellate Lipids

When *Z. nutricula* cultures living symbiotically with *Collozoum pelagicum* of the order Collodaria were placed under thermally stressful conditions, dinoflagellate density decreased, and dinoflagellate organelles were damaged (Villar et al., 2018). However, even while under thermal stress, the dinoflagellates in symbiosis with this polycystine radiolarian were still capable of photosynthetic functions (Villar et al., 2018). Although researchers have determined how *Z. nutricula* responds to thermal stress while in

symbiosis with Collodaria in terms of chloroplast functionality, little is known about the actual lipid content of *Z. nutricula* under normal conditions. Since chloroplasts within symbiotic cells of *Z. nutricula* remained intact and functionally efficient during thermal stress while other cellular components experienced degradation, the dinoflagellate lipid composition could provide insight about the types of chloroplast lipids structurally supporting photosynthetic membranes and photosystems in *Z. nutricula*.

Marine and freshwater dinoflagellates are composed of a variety of lipids that aid in proper functioning of the cell. Dinoflagellate cellular membrane lipids include glycolipids, betaine lipids, and phospholipids (Leblond et al., 2015; Leblond & Dahmen, 2016). Glycolipids such as monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) function by providing structure for the thylakoid membrane in photosynthetic chloroplasts, which in turn provides structure for chloroplast photosystems (Kobayashi, 2016). Reductions in the integrity of the photosystems caused by factors such as light stress can result in photoinhibition, reduced photosynthetic activity due to light (Downs et al., 2013; Hölzl et al., 2009). Phospholipids are cellular lipids that provide structural support for the cell (Kumari et al., 2013). Betaine lipids are non-phosphorous-containing analogs of phospholipids that serve as cellular lipids, and they are important in phosphorus-deficient cells because betaine lipids increase in quantity to compensate for a decrease in phospholipid quantities which can occur during phosphate starvation (Murakami et al., 2018). It is theorized that phytoplankton in phosphorous-lacking oligotrophic environments contain more betaine lipids than phytoplankton in eutrophic environments (Van Mooy et al., 2009). This could be true for *Z. nutricula* since it is symbiotic with radiolarians in oligotrophic environments.

Sterols, which play an important role in cell membrane fluidity and permeability, are also an important cellular lipid in dinoflagellates (Dawaliby et al., 2015; Freeman et al., 1976). Some dinoflagellates have been found to contain modified sterols that are believed to protect against predators because the sterols are nutritionally poor and are insufficient to sustain the predator (Giner et al., 2003). In addition, sterols may also provide protection from toxins because specific sterols may bind to the toxin, and thus hinder toxin functionality (Deeds & Place, 2006). As a result, this defense mechanism can serve to protect the dinoflagellate cell from toxins it produces (Deeds & Place, 2006). However, the protective function of toxins has only been studied in the toxic dinoflagellate *Karlodinium micrum* which is synonymous with *K. veneficum* (D.Ballantine) J.Larsen (Deeds & Place, 2006). Many distinct sterols are produced by different species of dinoflagellates due to the varying side chains and characteristics of the sterol ring structure, and the different sterols produced can be affected by alterations in environmental conditions such as heat and light (Volkman, 2003; Withers, 1983). *Z. nutricula* is taxonomically within the class Dinophyceae, and it has been determined that dinosterol (4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol), a major sterol for this class because it is produced by many (but not all dinoflagellates), can be used as a dinoflagellate biomarker in marine sediments (Volkman et al., 1993; Volkman, 2003). However, the sterol composition of *Z. nutricula* has never been examined.

Lipids are important for dinoflagellate and dinoflagellate host survival because lipids provide necessary stability for cellular components (e.g. cell membrane) and processes (e.g. photosynthesis). The purpose of this research is to analyze the lipid content of the dinoflagellate *Z. nutricula*, with emphasis placed on those classes of lipids,

sterols and chloroplast-associated glycolipids, which after decades of research are the best characterized lipids within the class Dinophyceae. Research has previously not determined the specific sterols and glycolipids produced by *Z. nutricula* as a radiolarian symbiont. Thus, this research will provide valuable baseline information regarding what lipids are produced and stored in the organism, and how such lipids could affect symbiotic relationships with radiolarians. Additionally, this research will give insight into how *Z. nutricula* is taxonomically related to other dinoflagellates as based on lipid composition.

Methods

Zooxanthella *Culture and Growth Conditions*

Zooxanthella nutricula CCMP 3427 was acquired from the National Center for Marine Algae and Microbiota (East Boothbay, ME; previously known as the Center for the Culture of Marine Phytoplankton). This culture is synonymous with culture collection strain RCC 3387 originally isolated from Villefranche-sur-Mer Bay in France and originally housed at the Roscoff Culture Collection (Roscoff, France). *Z. nutricula* was autotrophically grown to exponential phase in quadruplicate in 2 L of L1 growth medium (Guillard & Hargraves, 1993) with a salinity of 35 psu. The cultures were grown at 20°C under a 14/10 h light/dark cycle at an irradiance of approximately 50 μmol photons/$m^2 \cdot s^1$.

Cell Harvesting and Lipid Extraction

After sufficient growth of the cultures at 20°C in which cells were at a concentration of roughly 10^4 cells/mL, the cells were harvested using filtration onto Whatman 934-AH glass microfiber filters (GE Healthcare, Chicago, IL, USA). Lipids were extracted from the harvested cells using the techniques of Leblond and Chapman (2000).

Lipid Fractionating

Lipid classes were separated based on polarity using column chromatography using the methods described by Leblond and Chapman (2000). To separate the lipids, the columns used activated Unisil silica (1.0 g, 100-200 mesh, activated at 120°C, Clarkson

Chromatography, South Williamsport, PA, USA). The following describes the solvents used to separate the lipids into 5 fractions with increasing polarity: Fraction 1) 12 mL methylene chloride (sterol esters), Fraction 2) 15 mL 5% acetone in methylene chloride with 0.05% acetic acid (free sterols, tri- and diacylglycerols, and free fatty acids), Fraction 3) 10 mL 20% acetone in methylene chloride (monoacylglycerols), Fraction 4) 45 mL acetone (MGDG and DGDG), and Fraction 5) 15 mL methanol with 0.1% glacial (polar lipids, phospholipids, and betaine lipids).

Sterol Processing and Analysis

Sterol esters and free sterols from Fractions 1 and 2 were saponified and derivatized to form trimethylsilyl (TMS)-ether derivatives as described by Leblond and Chapman (2002). Gas chromatography/mass spectrometry (GC/MS) with positive-ion electron impact (EI) was used to analyze the sterol derivatives using the GC/MS conditions described by Khadka, Salem, and Leblond (2015). The following conditions were used: 1 μl of the sample was injected into the GC/MS in a splitless manner with the injector set at 280°C, the transfer line set at 275°C, the helium carrier at 28 cm/sec, 70eV with a scanning range of 50-600 amu and a cycle time of 1.1 sec. The temperature of the GC was held at 50°C for 1 minute, increased by 15°C per minute until reaching 170°C, and increased by 10°C per minute until reaching 300°C where it held for 11 minutes. Retention times (RT) were used to calculate relative retention times (RRT) to cholesterol based on the methods of Jones et al. (1994). The TMS-ether derivative was compared to a 22-dehydrocholesterol (cholesta-5,22Z-dien-3β-ol) standard that was acquired from Steraloids (Newport, Rhode Island). Comparisons for other sterols were compared to data

published by Jones et al. (1994) and sterol standards including 7-dehydrocholesterol (cholesta-5,7-dien-3β-ol), stigmasterol (5,22-cholestadien-24β-ethyl-3β-ol), and desmosterol (cholesta-5,24-dien-3β-ol) from Sigma-Aldrich (St. Louis, Missouri), brassicasterol (24-methylcholesta-5,22E-dien-3β-ol), fucosterol (5-cholesten-24(28)-ethylidene-3β-ol), and 5α-cholesta-8,24-dien-3β-ol from Steraloids (Newport, Rhode Island), β-sitosterol (5-cholesten-24β-ethyl-3β-ol) with campesterol (24-methylcholesta-5-en-3β-ol) from TCI America (Portland, Oregon), and cholesterol from an unknown source.

Galactolipid Processing and Analysis

Galactolipids from Fraction 4 were dissolved in a solvent mixture of methanol, chloroform, and 50 mM sodium acetate based on the methods of Welti et al. (2002) to produce positively charged sodium adducts [$M+Na^+$]. The adducts were scanned using positive-ion electrospray/mass spectrometry (ESI/MS) from 100-2,000 amu through direct injection of a 5 μl sample into a methylene chloride carrier solvent at 0.5 ml/min into a Finnigan DecaXP ion trap mass spectrometer (Waltham, MA, USA) based on the methods of Gray et al. (2009). The ESI/MS was calibrated before runs using Pierce LTQ ESI positive ion calibration solution (catalog number 88322, Thermo Fisher Scientific, Waltham, MA, USA). After isotopic correction for ^{13}C forms, the relative abundance of each galactolipid was identified. Using the total intensity of all galactolipid ions, the relative percentage distributions were identified using the methods of Gray et al. (2009). Further ESI/MS/MS scans were conducted on certain galactolipids by using a collision energy between 37.5 and 48%. Differences in the mass of original ions and their

fragments were used to identify the major cleaved fatty acids. The position of the acyl chain as either *sn*-1 or *sn*-2 was identified based on the relative percent compositions according to the procedure of Gray et al. (2009) as based on an earlier procedure created by Guella et al. (2003).

Results

Sterols

Fifteen sterols as TMS derivatives and one steroidal ketone were found in *Z. nutricula*, and of these sterols and steroidal ketones, eight were identifiable, as shown in Table 1. Major sterols included 22-dehydrocholesterol (cholesta-5,22E-dien-3β-ol), dinosterol, dinostanol (4α,23,24-trimethyl-5α-cholestan-3β-ol), and dinostanone (4α,23,24-trimethyl-5a-cholestan-3-one). Sterol identification was determined by comparing the masses of major molecular ion fragments with those of previously identified TMS derivatives of sterols from Jones et al. (1994) and other works as noted, as well as authentic standards where available. Examples of mass spectral characterization of major sterols and a steroidal ketone are discussed below.

The sterols were classified as either free sterols or sterol esters, as shown in Table 1. The interfraction comparison between free and esterified sterols showed that the free sterol fraction accounted for the majority of the total sterols (76.6 ± 8.7%). The most abundant free sterol, 22-dehydrocholesterol, accounted for 43.4 ± 2.5% of the free sterols and was a minor constituent of the sterol ester fraction with an abundance of 3.2 ± 2.3%. Other prominent free sterols included dinosterol which accounted for 25.5 ± 3.3% of the free sterols and dinostanol which accounted for 10.0 ± 0.4% of the free sterols. These three free sterols accounted for 78.9% of the free sterol fraction. Cholesterol accounted for a very low overall abundance of the free sterols with an abundance of 0.4 ± 0.1% and was not present as a sterol ester. The most abundant sterol ester accounting for 60.0 ± 4.1% of the sterol esters was an unknown $C_{29:1}$ sterol that was not present as a free sterol. The second most prominent sterol ester with an abundance of 25.3 ± 6.0% was

dinostanone. The unknown $C_{29:1}$ sterol and dinostanone accounted for 85.3% of the sterol ester fraction.

The mass spectrum of 22-dehydrocholesterol displayed major fragments at *m/z* 441 (M^+-CH_3), 366 (M^+-TMS-O-H), 351 (M^+-TMS-O-H-CH_3), 327 (M^+-TMS-O-H-C_3H_4), and 255 (M^+-TMS-O-H-side chain). These fragments were consistent with those of the 22-dehydrocholesterol standard's mass spectrum data shown in Figure 1. The side chain stereochemistry of 22-dehydrocholesterol was determined based on relative retention time (RRT) values since major fragments reported by Jones et al. (1994) were the same for both the *cis* (Z) and *trans* (E) stereoisomers. The RRT of the isomer isolated from *Z. nutricula* was 0.901, and according to Jones et al. (1994), the RRT of the *cis* isomer was 0.83 and the RRT of the *trans* isomer was 0.90. We aimed to compare the RRT value of the *trans* isomer standard with that of the isomer isolated in our study, but the standard was commercially unavailable. Nonetheless, RRT values for other sterols found in our study were comparable to those from Jones et al. (1994) apart from 4α,24-dimethyl-5α-cholest-22E-en-3β-ol which was presented by Jones et al. (1994) with two conflicting RRT values of 1.41 and 1.47. However, Mansour et al. (1999), Leblond and Chapman (2004), and Thomson et al. (2004) presented the RRT value of 4α,24-dimethyl-5α-cholest-22E-en-3β-ol as 1.47 which was consistent with our data, and they also presented RRT values for other sterols that were consistent with those from our study. Since the RRT values of sterols in our study were similar to those from Jones et al. (1994), the 22-dehydrocholesterol isomer identified in our study was determined to be the *trans* isomer given that its RRT value was 0.901.

The spectrum of dinosterol displayed major fragments at *m/z* 485 (M^+-CH_3), 388 (M^+-C_8H_6), 359 (M^+-2H-side chain), and 271 (M^+-TMS-O-H-side chain) which were consistent with those of the dinosterol mass spectra data provided by Atwood et al. (2014). The mass spectrum of dinostanol displayed major fragments at *m/z* 487 (M^+-CH_3), 412 (M^+-TMS-O-H), 397 (M^+-TMS-O-H-CH_3), and 373 (M^+-C_9H_{21}) which were consistent with those of the dinostanol mass spectrum data provided by Piretti et al. (1997). The mass spectrum of dinostanone displayed major fragments at *m/z* 413 (M^+-CH_3), 331 (M^+-C_7H_{13}), and 245 (M^+-$C_{13}H_{27}$) which were consistent with those of the dinostanone mass spectrum data provided by Mansour et al. (1999).

Galactolipids

Structurally, galactolipids have two fatty acids (acyl chains) attached to a single galactose or digalactose sugar moiety (Guella et al., 2003). The acyl chains in a galactolipid molecule can be the same (e.g. 18:5/18:5) or different (e.g. 18:5/18:4). The 18:5 fatty acid is octadecapentaenoic acid which contains 18 carbons and 5 double bonds (Figure 3). The 18:4 fatty acid is octadecatetraenoic acid which contains 18 carbons and 4 double bonds (Figure 3). The first double bond from the methyl end of both 18:5 and 18:4 fatty acids are in the n-3 position. Three galactolipids were found in *Z. nutricula* and were identified as 18:5/18:5 MGDG (*sn*-1/*sn*-2), 18:5/18:4 MGDG, and 18:5/18:4 DGDG, as shown in Table 2 and Figure 3. Stereospecific numbering (*sn*) identifies the placement of the acyl chains according to which carbon of the glycerol backbone the chains attach (Guella et al., 2003). The terminal carbons of glycerol include carbons 1 and 3, and carbon 2 is positioned between them as depicted in Figure 3.

The galactolipids 18:5/18:4 MGDG (*sn*-1/*sn*-2) and 18:5/18:4 DGDG required differentiation of the attached fatty acids based on *sn* position. Positive-ion ESI/MS/MS analysis identified the acyl chains as either *sn*-1 or *sn*-2 because the strongest peak represented the acyl chain in the *sn*-2 position due to the cleavage of the *sn*-1 acyl chain (Guella et al., 2003). For both of the galactolipids, the 18:5 acyl chain attached to the *sn*-1 position, and the 18:4 acyl chain attached to the *sn*-2 position since cleavage of the 18:5 acyl chains resulted in the strongest peaks (Figure 3).

The most prominent galactolipid was 18:5/18:5 MGDG with an abundance of 42.0 ± 1.1%. The second major galactolipid was 18:5/18:4 DGDG with an abundance of 33.5 ± 3.3%, and the third galactolipid, 18:5/18:4 MGDG, had an abundance of 24.4 ± 2.5%. All galactolipids in *Z. nutricula* were C_{18}/C_{18} forms of MGDG and DGDG.

Table 1. Sterols found in the free sterol and sterol ester fractions of *Zooxanthella nutricula* CCMP 3427.

Carbon Number	Suggested Structure	Molecular Weight[a]	Retention Time (min)	RRT[b]	Average Relative Abundance of Free and Esterified Sterols (%)	Average Relative Abundance of Total Sterols (%)
C_{27} Sterols	Unidentified $C_{27:2}$ sterol	456	36.9	0.838	0.2 ± 0.2	0.2
	Cholesta-5,22E-dien-3β-ol (22-dehydrocholesterol)	456	37.15	0.901	43.4 ± 2.5 (3.2 ± 2.3)	33.9
	Unidentified $C_{27:1}$ sterol	456	37.22	0.919	1.1 ± 0.5 (0.9 ± 0.5)	1.1
	Cholest-5-en-3β-ol (cholesterol)	458	37.57	1	0.4 ± 0.1	0.3
C_{28} Sterols	Unidentified $C_{28:1}$ sterol	472	37.83	1.083	5.5 ± 1.5 (2.0 ± 1.4)	4.7
	Unidentified $C_{28:1}$ sterol	472	37.93	1.11	0.9 ± 0.2 (1.0 ± 0.5)	0.9
	24-Methylcholesta-5,22E-dien-3β-ol[c] (*R*-brassicasterol/*S*-crinosterol)	470	38.07	1.148	2.7 ± 0.6 (0.2)	2.1
	Unidentified $C_{28:1}$ sterol	472	38.33	1.218	5.3 ± 3.6	4.1
C_{29} Sterols	Unidentified $C_{29:1}$ sterol	486	39	1.398	0.6 ± 0.2 (2.3 ± 1.0)	1.0
	4α,24-Dimethyl-5α-cholest-22E-en-3β-ol	486	39.29	1.476	2.3 ± 0.3 (1.4 ± 0.8)	2.1
	4α,24-Dimethyl-5α-cholestan-3β-ol (4,24-dimethylcholestanol)	488	40.13	1.701	1.0 ± 1.0	0.8
	Unidentified $C_{29:1}$ sterol	488	40.19	1.718	(60.0 ± 4.1)	14.1

Table 1 Continued

Carbon Number	Suggested Structure	Molecular Weight[a]	Retention Time (min)	RRT[b]	Average Relative Abundance of Free and Esterified Sterols (%)	Average Relative Abundance of Total Sterols (%)
	4α,23,24-Trimethyl-5α-cholest-22E-en-3β-ol (dinosterol)	500	40.46	1.79	25.5 ± 3.3 (2.9 ± 1.1)	20.2
	4α,23,24-Trimethyl-5α-cholestan-3-one (dinostanone)	428	41.38	2.032	(25.3 ± 6.0)	5.9
	4α,23,24-Trimethyl-5α-cholestan-3β-ol (dinostanol)	502	41.52	2.075	10.0 ± 0.4 (0.9 ± 0.3)	7.9
Unclear	Unclear	Unclear	39.08	1.419	0.9 ± 0.5	0.7

[a] Molecular weight as sterol as a TMS derivative.
[b] Relative retention time to the TMS derivative of cholesterol.
[c] C_{24} stereochemistry not determined.
[d] Values in parentheses represent sterol esters.

Table 2. Relative abundance (in % of total fragment height using listed masses) of *Zooxanthella nutricula* CCMP 3427 galactolipids as determined via positive-ion ESI/MS/MS.

Galactolipid	Mass (*m/z*)[1]	Average Relative Abundance (%)
18:5/18:5 MGDG	789	42.0 ± 1.1
18:5/18:4 MGDG	791	24.4 ± 2.5
18:5/18:4 DGDG	953	33.5 ± 3.3

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

Discussion

Phylogenetic Placement of Z. nutricula *Within the Dinophyceae*

Dinoflagellates are a phylogenetically diverse group of protists that can be placed in multiple phylogenetic groups based on various characteristics such as the presence of plastids and thecal plates, and the tendency to form symbiotic relationships (Not et al., 2012). *Oxyrrhis marina* Dujardin is a heterotrophic (proto)dinoflagellate typically placed at the base of the dinoflagellate phylogenetic tree (Medlin & Cembella, 2013; Moestrup & Daugbjerg, 2007). Roughly half of all dinoflagellates are heterotrophic while the other half are autotrophic and contain plastids (Not et al., 2012). Since there are multiple types of dinoflagellate plastids, dinoflagellates can be separated into groups based on the plastid that they contain (Moestrup & Daugbjerg, 2007). The most common type of plastid among dinoflagellates contains the carotenoid pigment peridinin which is found in dinoflagellates of the orders Peridiniales, Gonyaulacales, Gymnodiniales, and Suessiales, among others (Moestrup & Daugbjerg, 2007; Not et al., 2012). The peridinin plastid is considered a secondary plastid because it arose from a red algal plastid through endosymbiosis (Moestrup & Daugbjerg, 2007). While peridinin has not been reported in *Z. nutricula*, phylogenetic analysis places *Z. nutricula* within the order Peridiniales which suggests that it does contain peridinin (Probert et al., 2014), and *Z. nutricula* has the distinctive brownish red coloration of peridinin-containing dinoflagellates (J. Graeff, personal observation).

Other autotrophic dinoflagellates lack the peridinin plastid and instead contain different secondary plastids or plastids that arose from subsequent endosymbiosis events that resulted in tertiary plastids (Dorrell & Howe, 2015). Characteristics of secondary and

tertiary plastids include additional plastid membranes and different types of chlorophyll and pigments than those in the original plastid (Dorrell & Howe, 2015). Fucoxanthin is a pigment in the peridinin-lacking tertiary plastid that originated from haptophyte algae, and this plastid is found in *Karenia* and *Karlodinium* species (Hackett et al., 2004; Tengs et al., 2000; Yoon et al., 2005). Another aberrant plastid is found in *Lepidodinium* species, and it lacks both peridinin and fucoxanthin and is derived from green algae through secondary endosymbiosis (Keeling, 2010; Matsumoto et al., 2011; Watanabe et al., 1991).

The presence of a cellulose thecal plate, and the pattern of the plates can be used as a morphologic trait to reinforce phylogeny (discussed below; Hackett et al., 2004; Medlin & Cembella, 2013). Research about the mechanics of dinoflagellate thecal plates revealed that the plates are important structures since they provide protection from predators and rough waters (Lau, 2007). Dinoflagellates within the orders Peridiniales and Gonyaulacales have thecal plates; however, Gymnodiniales and Suessiales are notable orders that lack thecal plates (Not et al., 2012). *Z. nutricula* contains a series of six thecal plates and has a unique arrangement of a single plate that is uncommon in the Peridiniales but is found in a few heterotrophic peridinioid dinoflagellates (Probert et al., 2014). Overall, the arrangement of the plates around the cell supports the classification of *Z. nutricula* as a discrete peridinioid species (Probert et al., 2014). Based on thecal plate arrangement and rRNA gene analysis, *Z. nutricula* is closely related to *Heterocapsa* spp., *Scrippsiella* spp., *Ensiculifera* spp., *Pentapharsodinium* spp., and *Azadinium* spp. (Probert et al., 2014). *Z. nutricula* is a symbiont of the radiolarian order Collodaria, and *Heterocapsa* sp., *Scrippsiella* sp., and *Azadinium* sp. have been observed as symbionts of

the radiolarian order Acantharia (Decelle et al., 2012). As such, *Z. nutricula* is closely related to other peridinioid species that form symbiotic relationships with radiolarians (Probert et al., 2014). However, these dinoflagellates are distantly related to *Symbiodinium* within the order Suessiales (Probert et al., 2014). Therefore, as based on the morphology of thecal plates, *Z. nutricula* is more closely related to species that form symbiotic relationships with radiolarians than species that form symbiotic relationships with cnidarians.

Overall, there are major groups of dinoflagellates based on different phylogenetic orders due to phylogenetic characteristics that often provide a basis for grouping. While further research is needed regarding the phylogeny of *Z. nutricula*, it has been determined based on rRNA sequences that the genus *Zooxanthella* is part of the order Peridiniales rather than Suessiales (Gottschling & Mclean, 2013; Probert et al., 2014). For continuity, this paper will discuss the phylogeny of *Z. nutricula* as it relates to the Peridiniales. This strain of *Z. nutricula*, CCMP 3427/RCC 3387, is closely related to the species of *Heterocapsa* and *Ensiculifera* based on small subunit rRNA gene and large subunit rRNA gene analysis, respectively (Probert et al., 2014).

The genus *Heterocapsa* includes twenty accepted species that collectively possess similar physical traits such as thecal plate arrangement and chloroplast placement, but body scales are important features that have been used to differentiate between species of this genus (Attaran-Fariman & Javid, 2013; Guiry & Guiry, 2020; Iwataki, 2008; Salas et al., 2014). *H. triquetra* which is synonymous with *Kryptoperidinium triquetrum* (Ehrenberg) U.Tillmann, M.Gottschling, M.Elbrächter, W.-H.Kusber & M.Hoppenrath, *H. rotundata* (Lohmann) Gert Hansen, *H. niei* (Loeblich III) L.C.Morrill & Loeblich III,

H. pygmaea Loeblich III, R.J.Schmidt & Sherley, *H. illdefina* (Herman & Sweeney) L.C.Morrill & Loeblich III, and *H. circularisquama* are prominent species of *Heterocapsa* and have thus been the most phylogenetically researched since a few species are rare or are only found in certain parts of the world (Iwataki, 2008; Orr et al., 2012; Yoshida et al., 2003; Zhang et al., 2007). Although phylogenetic analyses have determined the placement of *Heterocapsa* species as they relate to each other, the phylogenetic placement of *Heterocapsa* as related to other genera is unresolved (Tillmann et al., 2017; Zhang et al., 2007). The classification of *Heterocapsa* within the Peridiniales is supported; however, more research is needed to determine its relation to other peridinioid species since earlier research classified *Heterocapsa* as basal to dinoflagellates due to its seemingly early divergence, but more recent research classifies *Heterocapsa* as a sister group to other peridiniod species (Janouškovec et al., 2016; Orr et al., 2012; Zhang et al., 2007). Within the genus *Heterocapsa*, *H. triquetra*, *H. rotundata*, *H. niei*, and *H. pygmaea* are closely related to *Z. nutricula* based on rRNA gene analysis (Probert et al., 2014). While *H. circularisquama* was not included within the phylogenetical tree of *Z. nutricula*, it is a notable species due to its production of toxins within HABs that are particularly harmful to bivalves (Iwataki, 2008; Horiguchi, 1995). *H. triquetra* and *H. rotundata* also cause algal blooms that result in fluctuations of dissolved oxygen levels, but these species do not cause harm to other environmental organisms via toxins (Lemley et al., 2018; Tas, 2015).

Sterols of Z. nutricula *in Relation to Other Dinoflagellates*

Previous research about the sterols present in different dinoflagellates provides a basis for dinoflagellates to be grouped according to their sterol composition, thereby allowing for comparisons to be made between phylogenetic groups (Leblond et al., 2010; Withers, 1983). Common dinoflagellate sterols include dinosterol, cholesterol, and 4,24-dimethylcholestanol (4,24-dimethyl-5α-cholestan-3β-ol; Withers, 1983). Dinoflagellates tend to produce 4α-methyl sterols such as dinosterol more than 4-desmethyl sterols such as cholesterol since 4α-methyl sterols function as end products in dinoflagellates (Kokke et al., 1980; Loeblich, 1984; Withers, 1983). Dinosterol tends to be the major sterol in many dinoflagellates, and thus is an important marine sediment biomarker (Volkman, 2003; Volkman et al., 1993; Withers, 1983). However, as an example of a group of dinoflagellates that produces signature 4α-methyl sterols other than dinosterol, *Amphidinium* species lack dinosterol and instead produce amphisterol (4α-methyl-5α-ergosta-8(14),24(28)-dien-3β-ol) as the major sterol which can also function as a biomarker (Withers, 1983).

Since many dinoflagellates engage in symbiotic relationships with marine organisms, gorgosterol is also common among zooxanthellae dinoflagellates because the production of gorgosterol increases within symbiont-host interactions but can decrease when the symbiont and host are no longer interacting with each other (Giner & Djerassi, 1991; Withers, 1983). Gorgosterol contains a unique side chain which is synthesized from dinosterol (Giner & Djerassi, 1991; Kumar & Chopra, 2005). Gorgosterol has been found in cnidarians, and it is believed that zooxanthellae are responsible for synthesizing gorgosterol from dinosterol and transferring gorgosterol to the host (Steudler et al., 1977;

Withers et al., 1982). Therefore, gorgosterol can serve as a biomarker for zooxanthellae dinoflagellates engaged in symbiosis (Ciereszko, 1989). Although gorgosterol is found in many cnidarians and their symbiotic dinoflagellates, the function of gorgosterol is unknown (Rampen et al., 2009). Determining its function could provide insight into symbiotic relationships and could identify what stimulates dinoflagellates to produce gorgosterol while in symbiosis with cnidarians (Withers et al., 1982). Gorgosterol was not identified in cultures of *Z. nutricula* grown *ex hospite*; however, future research could determine if gorgosterol is produced when this symbiont is examined *in hospite*. Also, while cnidarians and the radiolarian order Acantharia can simultaneously host multiple species of dinoflagellate symbionts, it is unknown if other radiolarian orders simultaneously host multiple symbionts and if co-symbionts of *Z. nutricula* could produce gorgosterol (Carlos et al., 2000; Decelle et al., 2012; Rowan & Knowlton, 1995).

Cholesterol is found in many dinoflagellates and is also common in zooxanthellae, but the abundance of cholesterol can vary between species (Loeblich, 1984; Volkman, 1986). Cnidarians contain a high abundance of cholesterol, and symbiotic dinoflagellates have an important role of providing cholesterol for the cnidarian host (Hambleton et al., 2018; Nelson et al., 2000). However, the role of cholesterol in dinoflagellate-radiolarian symbiosis is unknown, and in *Z. nutricula*, cholesterol was found to be a minor sterol. The major sterol of *Z. nutricula*, 22-dehydrocholesterol, is structurally similar to cholesterol and 7-dehydrocholesterol, and some unrelated animals are capable of converting 7-dehydrocholesterol to cholesterol and ergosterol (ergosta-5,7,22-trien-3β-ol) to 22-dehydrocholesterol (Clark & Bloch, 1959; Wilton et al., 1966). However, the ability of cnidarians and radiolarians to perform these

biotransformations is unknown. While dinoflagellate species can produce numerous types of sterols, half of the sterol content of a given species is usually made up of two major sterols that vary by species (Withers, 1983). Minor sterols account for less than 10% of the total sterols and are typically chemical intermediates without a specific function within dinoflagellate cells (Loeblich, 1984). Major sterols typically include dinosterol, cholesterol, and 4,24-dimethylcholestanol (Leblond & Chapman, 2002). Minor sterols encompass a large variety of sterols and depend on the species, but they can include cholestanol (5α-cholestan-3β-ol), 24-methylcholesterol (24-methylcholest-5-en-3β-ol), 24-methyl-5α-cholestan-3β-ol, and 4α,23,24-trimethyl-5α-cholest-7-en-3β-ol (Leblond & Chapman, 2002).

This research aimed to identify the sterols of *Z. nutricula* and to use sterols to clarify the relatedness between *Z. nutricula* and other dinoflagellates. *Heterocapsa circularisquama* is a marine dinoflagellate within the order Peridiniales and is phylogenetically related to *Z. nutricula* (Horiguchi, 1995; Probert et al., 2014). The most abundant sterol in *H. circularisquama* was dinosterol which accounted for 39.5% of the total sterol content (Kaku & Hiraga, 2003). While dinosterol was the predominant sterol in *H. circularisquama*, this research shows that dinosterol was the second most predominant sterol in *Z. nutricula*, accounting for 20.2% of the total sterols, as shown in Table 1. Dinostanol was the second most abundant sterol in *H. circularisquama* (13.7%; Kaku & Hiraga, 2003) and was the fourth most abundant sterol in *Z. nutricula* and comprised 7.9% of the total sterols (Table 1). Interestingly, while cholesterol is common among dinoflagellates, both *H. circularisquama* and *Z. nutricula* contained relatively low amounts of cholesterol since it accounted for only 1% of the total sterols in *H.*

circularisquama (Kaku & Hiraga, 2003) and 0.3% of the total sterols in *Z. nutricula*. The major sterol of *Z. nutricula* was 22-dehydrocholesterol which was not present in *H. circularisquama* (Kaku & Hiraga, 2003). However, *H. circularisquama* contained 7-dehydrocholesterol at an abundance of 9.4% (Kaku & Hiraga, 2003). 7-Dehydrocholesterol differs from 22-dehydrocholesterol found in *Z. nutricula* due to the different structural placement of a double bond. The low abundance of cholesterol in *Z. nutricula* could be related to the high abundance of 22-dehydrocholesterol which is structurally the same as cholesterol but with an additional double bond. Ten sterols were identified from *H. circularisquama*, and of these sterols, five were also found in *Z. nutricula*: cholesterol, dinosterol, dinostanol, 4α,24-dimethyl-5α-cholest-22-en-3β-ol, and 4,24-dimethylcholestanol (Kaku & Hiraga, 2003).

The sterol compositions of four other *Heterocapsa* species: *H. niei*, *H. pygmaea* (3 strains), *H. illdefina*, and *H. triquetra* (2 strains) were also determined (Alam et al.,1984). These species contained the identifiable sterols of dinosterol, cholesterol, dinostanol, 24-methylcholesterol, and 4,24-dimethylcholestanol (Alam et al., 1984). All four *Heterocapsa* species contained dinosterol as the most abundant sterol (40.6-61.5%; Alam et al., 1984). Dinosterol was a major sterol in *Z. nutricula* but was not the most abundant sterol. Of the seven strains analyzed, the second most abundant sterol was either 4,24-dimethylcholestanol (19.2-33.5%) or dinostanol (14.6-22.7%; Alam et al., 1984). In *Z. nutricula*, 4,24-dimethylcholestanol was present with an abundance of 0.8%, and dinostanol was present as the fourth most abundant sterol with an abundance of 7.9%. Both sterols had a low abundance compared to *Heterocapsa*. Despite the phylogenetic relatedness between *Heterocapsa* and *Z. nutricula*, the predominant sterol in

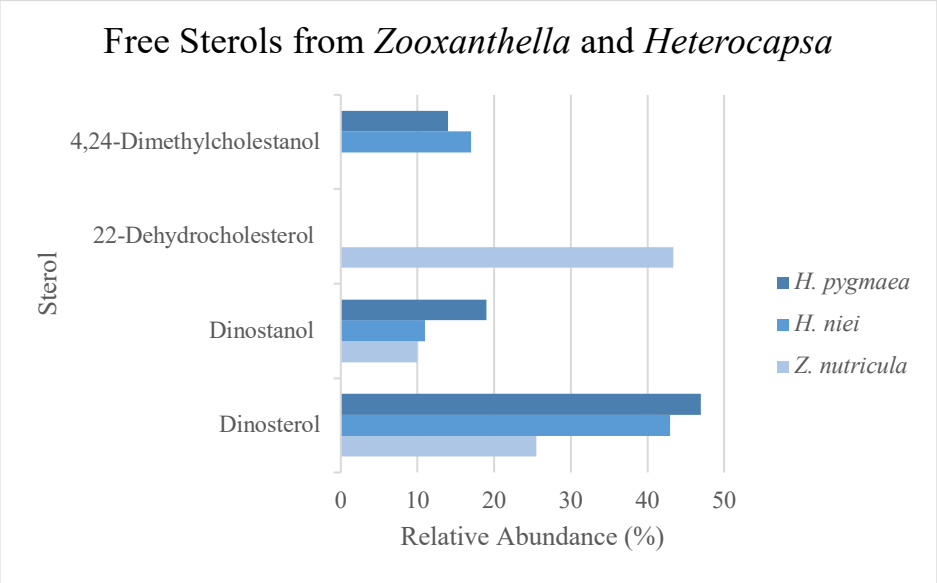
Z. nutricula, 22-dehydrocholesterol (33.9%; Table 1), was not present in the five *Heterocapsa* species previously analyzed.

The sterol compositions of *Heterocapsa* spp. reported by Kaku and Hiraga (2003) and Alam et al. (1984) did not differentiate between free sterols and sterol esters; however, another study did distinguish between free and esterified sterols of *H. niei* and *H. pygmaea* (Leblond & Chapman, 2002). Comparisons of free sterols and sterol esters between the dinoflagellate species *H. pygmaea*, *H. niei,* and *Z. nutricula* are shown in Figure 4. For both *H. niei* and *H. pygmaea*, dinosterol (43-47%) was the predominant free sterol and was not present as a sterol ester (Leblond & Chapman, 2002). Similarly, dinosterol was the second major free sterol (25.5%) in *Z. nutricula* and was found at a low abundance as a sterol ester (2.9%). Also, dinostanol was present as a free sterol in similar abundances in *H. niei* (11%) and *Z. nutricula* (10.1%) and was also present in *H. pygmaea* at a slightly higher abundance (19%; Leblond & Chapman, 2002). In both *Heterocapsa* species, dinostanol was not present as a sterol ester, and was found at a low abundance in *Z. nutricula* as a sterol ester (0.9%). Again, cholesterol was a minor free and esterified sterol in *H. niei* and *H. pygmaea* (Leblond & Chapman, 2002) and was also a minor free sterol in *Z. nutricula* while absent as a sterol ester. The major free sterol in *H. niei* and *H. pygmaea* was dinosterol, and the major sterol ester for *H. niei* was 4α,23,24-trimethyl-5α-cholest-24(28)-en-3β-ol while the major sterol ester for *H. pygmaea* was an unidentified C_{31} sterol (Leblond & Chapman, 2002; Figure 4). In contrast, the major free sterol in *Z. nutricula* was 22-dehydrocholesterol, and the major sterol ester was an unidentified $C_{29:1}$ sterol (Figure 4). Also, dinostanone was an abundant steroidal ketone in *Z. nutricula* but was not present in *Heterocapsa* spp. While the free

and esterified sterols of *H. circularisquama*, *H. illdefina*, and *H. triquetra* cannot be compared to *Z. nutricula*, data for *H. niei* and *H. pygmaea* provide insight into how sterols exist as free and esterified among these closely related dinoflagellates.

The phylogenetic tree of *Z. nutricula* identifies the species *Ensiculifera aff. loeblichii*, *E. cf. loeblichii*, and *E. aff. imariensis* as close relatives of *Z. nutricula* (Probert et al., 2014). The genus *Ensiculifera* was formed following the discovery of *E. mexicana* Balech; however, after inconsistencies with thecal plate arrangement, some *Ensiculifera* species were placed in the genera of *Scrippsiella* or *Pentapharsodinium* (Matsuoka et al., 1990). As a result, *E. loeblichii* E.R.Cox & H.J.Arnott was moved to the genus *Pentapharsodinium*, but this is now regarded as a misapplied name (D'Onofrio et al., 1999). However, *E. loeblichii* is accepted as a synonym of *Peridinium loeblichii* (E.R.Cox & H.J.Arnott) Dale (Cox & Arnott, 1971). *Ensiculifera* is a genus phylogenetically related to *Z. nutricula*, but no known research exists for the sterol composition of *Ensiculifera* or species synonymous with those in this genus since cultures are not available. Future research about *Ensiculifera* could help to clarify the phylogenetic relationship between *Ensiculifera* and *Z. nutricula* based on sterol composition.

22-Dehydrocholesterol was not present in *Heterocapsa* spp. although they are closely related to *Z. nutricula,* and its presence is unknown in the also closely related *Ensiculifera* spp.; however, 22-dehydrocholesterol is present in other dinoflagellate species. While the phylogenetic relationship between *Z. nutricula* and the genus *Gonyaulax* has not been studied directly, phylogenetic trees of *Gonyaulax* place *H. triquetra* as an outgroup (Ellegaard et al., 2003; Howard et al., 2009; Rhodes et al.,2006).



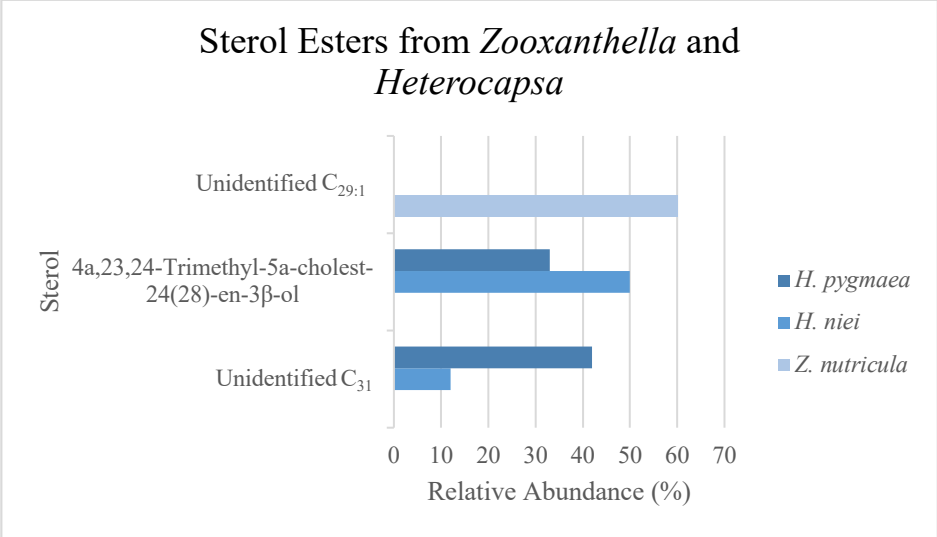


Figure 4. Comparison of select sterols from the free sterol and sterol ester fractions of *Heterocapsa pygmaea* CCMP 1322 and *Heterocapsa niei* UTEX 1564 (Leblond and Chapman, 2002) and *Zooxanthella nutricula* CCMP 3427.

Therefore, *Z. nutricula* is not closely related to *Gonyaulax* since *Z. nutricula* is closely related to *H. triquetra* (Probert et al., 2014). The total sterol composition of the distantly related *Gonyaulax polygramma* F.Stein contained 8.9% of 22-dehydrocholesterol as a free sterol and 8.7% of 22-dehydrocholesterol as a sterol ester (Volkman et al., 1984). The abundance of 22-dehydrocholesterol in *G. polygramma* remained relatively the same as a free sterol and as a sterol ester. However, in *Z. nutricula*, 22-dehydrocholesterol was the major free sterol (43.4%) but was a minor sterol ester (3.2%; Table 1).

As with *Gonyaulax*, the relation between *Z. nutricula* and the genus *Pyrocystis* has not been directly studied. However, based on phylogenetic trees, *Gonyaulax* is closely related to *Pyrocystis* which is expected due to similar characteristics between the two genera including reproductive bodies, nucleus shape, and bioluminescence (Elbrächter & Drebes, 1978; Zhang et al., 2007). However, *Pyrocystis* is not closely related to *Heterocapsa* as depicted by phylogeny based on rRNA genes (Orr et al., 2012). Therefore, *Pyrocystis* is not closely related to *Z. nutricula* since *Pyrocystis* is closely related to *Gonyaulax* and is not closely related to *Heterocapsa*. *Pyrocystis lunula* (Schütt) Schütt is a luminescent dinoflagellate, and 22-dehydrocholesterol in *P. lunula* accounted for 0.6% of the total sterol composition (Kokke et al., 1982). The abundance of 22-dehydrocholesterol in *P. lunula* and *G. polygramma* was very different than in *Z. nutricula*. While this particular sterol was found at a low abundance in dinoflagellates unrelated to *Z. nutricula*, it was absent from dinoflagellates related to *Z. nutricula*, specifically *Heterocapsa* spp.

Marine diatoms are also phytoplankton and among other differences, instead of having a cell wall composed of cellulose like dinoflagellates, diatoms have a cell wall

composed of silica (Seckbach & Kociolek, 2011). Diatoms and dinoflagellates typically differ in terms of sterol content since dinosterol is considered a biomarker for dinoflagellates and occurs in most dinoflagellate species (Withers, 1983). Although rare, dinosterol has also been found in the marine diatom *Navicula* sp., so some overlap of sterols occurs between diatoms and dinoflagellates (Volkman et al., 1993). 22-Dehydrocholesterol was reported to be found with high abundance in the diatoms *Fragilaria pinnata* (58.6%), *Thalassiothrix heteromorpha* (86.6%), and *Thalassionema nitzschioides* (47.7%; Barrett et al., 1995). 22-Dehydrocholesterol was also reported as the most predominant sterol in the diatom *Biddulphia sinensis* with an abundance of 70.1-81.7% of the total sterol content (Volkman et al., 1980). Despite the high abundance of 22-dehydrocholesterol in *B. sinensis*, which is of the order Biddulphiales, 22-dehydrocholesterol is rare in diatoms not within the order Bacillariales (Barrett et al., 1995; Serrazanetti et al., 2006; Volkman et al., 1980). The diatom *Cylindrotheca closterium* is of the order Bacillariales, and 22-dehydrocholesterol accounted for 49.5% of the total sterol content (Serrazanetti et al., 2006). After evaluation of the genus *Cylindrotheca*, it was concluded that *C. closterium* is synonymous with the diatom *Nitzschia closterium* (Reimann et al., 1964; Ryabushko et al., 2019). *N. closterium* was reportedly found near the central capsule of the radiolarian *Collozoum radiosum* belonging to the order Collodaria (Ishitani et al., 2012; Swanberg & Anderson, 1985). Collodaria symbionts are known to congregate around the central capsule at night, so it is possible that *C. closterium* is a symbiont of *C. radiosum* (Anderson et al., 1983; Suzuki & Not, 2015). However, no further research clarifies whether *C. closterium* is a radiolarian symbiont. If *C. closterium* is a symbiont of Collodaria, then 22-

dehydrocholesterol could be an important sterol for Collodaria since 22-dehydrocholesterol was the major sterol for both *C. closterium* and *Z. nutricula*.

22-Dehydrocholesterol is a predominant sterol in certain species of diatoms and accounts for a large percentage of the total sterols. However, 22-dehydrocholesterol does not appear to be a predominant sterol in dinoflagellates, and when it is produced, it accounts for only a small percentage of the total sterols except in the data presented for *Z. nutricula*. The dominance of 22-dehydrocholesterol in *Z. nutricula* suggests that *Z. nutricula* belongs in Cluster 3 of dinoflagellates when grouped based on sterol content (Leblond et al., 2010). Cluster 3 contains dinoflagellates such as *Gymnodinium* spp., *Polarella glacialis* M.Montresor, G.Procaccini, & D.K.Stoecker, *Lingulodinium polyedrum* (F.Stein) J.D.Dodge, and *Protoceratium reticulatum* (Claparède & Lachmann) Bütschli in which the most abundant sterols include *cis*-22-dehydrocholesterol (cholesta-5,22Z-dien-3β-ol), 24-methylcholesta-5,22E-dien-3β-ol, and 4,24-dimethylcholestanol (Leblond et al., 2010). However, *Heterocapsa* spp. are grouped within Cluster 6 along with *Scrippsiella* spp., *Pyrocystis* spp., and *Gymnodinium* spp. due to the high abundance of dinosterol and dinostanol (Leblond et al., 2010). The clustering for the genus *Ensiculifera* is unknown since data for the sterols of this species presently do not exist.

It is recognized that zooxanthellae dinoflagellates transfer sterols to cnidarian hosts, and it is believed that sterols are also transferred to radiolarian hosts (Anderson, 2012; Hambleton et al., 2018). The sterol composition of coral has been analyzed which identified cholesterol and 24-methylcholesterol as being major coral sterols (Kanazawa et al., 1976). In addition, non-canonical Niemann-Pick Type C2 (NPC2) proteins were identified in cnidarians and were shown to regulate the transfer of zooxanthellae-derived

sterols such as cholesterol to the cnidarian host (Hambleton et al., 2018). Blocking zooxanthellae sterols from transferring to the host threatened the symbiotic relationship which demonstrates the importance of zooxanthellae-derived sterols in cnidarian symbiosis (Hambleton et al., 2018). While extensive knowledge is known about dinoflagellate-cnidarian relationships, little is known about dinoflagellate-radiolarian relationships, especially about the transfer of sterols between organisms. Unlike with cnidarians, there is no known research about radiolarian sterols. Therefore, it is difficult to understand which sterols produced by symbiotic dinoflagellates are used by radiolarians. *Z. nutricula* has been isolated from the radiolarian *Collozoum inerme* from the order Collodaria, unidentified radiolarians from the orders Spumellaria and Nassellaria, and the radiolarian *Thalassicolla nucleata* (Liu et al., 2019; Probert et al., 2014; Zettler et al., 1999). The particular dinoflagellate strain used in this research was isolated from a spumellarian (Probert et al., 2014). Future research on the dynamics and exchanges between dinoflagellates and radiolarians, particularly Spumellaria, will provide insight on whether the major sterol of *Z. nutricula*, 22-dehydrocholesterol, is of importance to radiolarians as well as what sterols are transferred from *Z. nutricula* to the host, if any. Also, while gorgosterol, a biomarker for symbiotic dinoflagellates that is often transferred to the host, was not found in *Z. nutricula* when grown *ex hospite*, future research could determine if gorgosterol is present when this symbiont is examined *in hospite*. It is evident that dinoflagellates and cnidarians have a strong symbiotic relationship in which the host receives sterols from the symbiont. A similar relationship could exist between radiolarians and dinoflagellates, and research could help clarify the role of dinoflagellates in radiolarian symbioses.

Galactolipids of Z. nutricula *in Relation to Other Peridinin-Containing Dinoflagellates*

Galactolipids are found in photosynthetic membranes where they provide support for photosystems, so they are important lipids for photosynthetic dinoflagellates (Kobayashi, 2016; Kumari et al., 2013). Galactolipid analyses of 35 peridinin-containing dinoflagellates identified two main clusters of dinoflagellates based on the MGDG and DGDG produced by the dinoflagellates (Gray et al., 2009). The first cluster contained dinoflagellates that mainly produced C_{18}/C_{18} forms of MGDG and DGDG, and 18:5/18:4 DGDG was typically the major form of DGDG (Gray et al., 2009). The second cluster contained dinoflagellates that mainly produced C_{20}/C_{18} forms of MGDG and DGDG (Gray et al., 2009). Based on the galactolipid content of *Z. nutricula*, this dinoflagellate belongs in Cluster 1 due to the dominance of C_{18}/C_{18} forms of both MGDG and DGDG (Table 2). Also, 18:5/18:4 DGDG was the only form of DGDG identified in *Z. nutricula*, which is consistent with Cluster 1 since this form of DGDG tends to be the major form. The major forms of MGDG in *Z. nutricula*, 18:5/18:5 and 18:5/18:4, are also characteristic of the major forms of MGDG in Cluster 1 (Gray et al., 2009). However, in both Cluster 1 and 2, the analyzed dinoflagellates tended to contain more DGDG than MGDG, but the opposite was true for *Z. nutricula* (Gray et al., 2009). Other peridinin-containing dinoflagellates belonging to Cluster 1 include *Heterocapsa* and *Symbiodinium* species (Gray et al., 2009). While clusters based on dinoflagellate sterols have a phylogenetic relationship, clusters based on galactolipids do not appear to have a phylogenetic relationship (Leblond et al., 2010; Gray et al., 2009). However, there is a possible relationship between the galactolipids and ecology of dinoflagellates (Gray et al., 2009).

Little research has analyzed the fatty acid content of radiolarians. However, research about dinoflagellates living symbiotically with coral shows that symbiont fatty acids, such as 16:3(n-4, hexadecatrienoic acid), 16:4(n-1, hexadecatetraenoic acid), 18:3(n-6, octadecatrienoic acid), 18:4(n-3), and 22:6(n-3, docosahexaenoic acid), can be transferred to the host coral (Papina et al., 2003). Also, in cnidarian-dinoflagellate symbiotic relationships, it is proposed that cnidarian hosts may be able to transfer fatty acids to the symbiont or alter the synthesis of polyunsaturated fatty acids in the symbiont (Imbs et al., 2014). Further research about radiolarian fatty acids and the capability to synthesize fatty acids could clarify whether the 18:5 and 18:4 fatty acids found in *Z. nutricula* are transferred to the host, if the host is able to transfer fatty lipids to the symbiont, and if the host can change the fatty acids produced by the dinoflagellate while in symbiosis.

Conclusions

This research provides the first analysis of the sterol and galactolipid content of *Z. nutricula* in which the sterol 22-dehydrocholesterol was present at an unusually high abundance for dinoflagellates, and the galactolipids were dominated by C_{18}/C_{18} forms of MGDG and DGDG. The taxonomy of this species has been in question due to the multiple synonyms given to this dinoflagellate, and the sterol content does not closely resemble that of phylogenetically related species such as *Heterocapsa* spp. Therefore, future research of the sterol content of *Ensiculifera* species, particularly *E. loeblichii* and *E. imariensis,* once cultures are made available, could help clarify taxonomic relations with other dinoflagellates. Since cnidarians and *Symbiodinium* have a dynamic

relationship in which *Symbiodinium* produce cholesterol for the host, further research about radiolarian sterols and the diatom *C. closterium* as a radiolarian symbiont could clarify whether 22-dehydrocholesterol is important for radiolarian symbiosis. Also, research that identifies 22-dehydrocholesterol as a major sterol in a dinoflagellate species could provide insight into the taxonomic placement of *Z. nutricula*. While 22-dehydrocholesterol is not produced in dinoflagellates closely related to *Z. nutricula*, the fatty acid-containing galactolipids identified in *Z. nutricula* are consistent with those of a group of other peridinin-containing dinoflagellates that mainly produced C_{18}/C_{18} forms of MGDG and DGDG. Future research about radiolarian sterols and fatty acid-containing lipids should elucidate whether *Z. nutricula* is a sterol and fatty acid provider, how *Z. nutricula* functions with radiolarians, and how sterols and fatty acids produced by *Z. nutricula* are important in radiolarian symbiosis.

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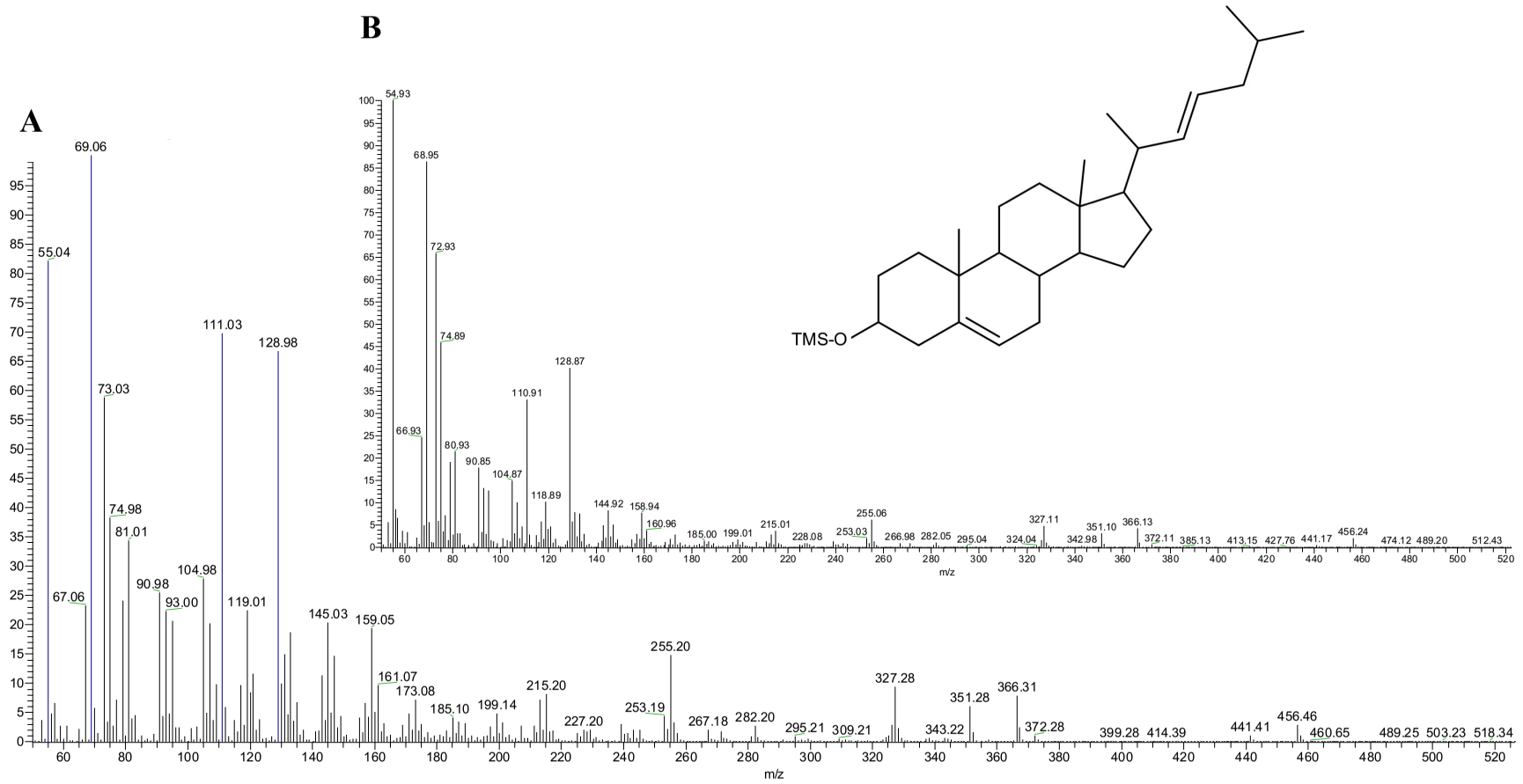
Appendix



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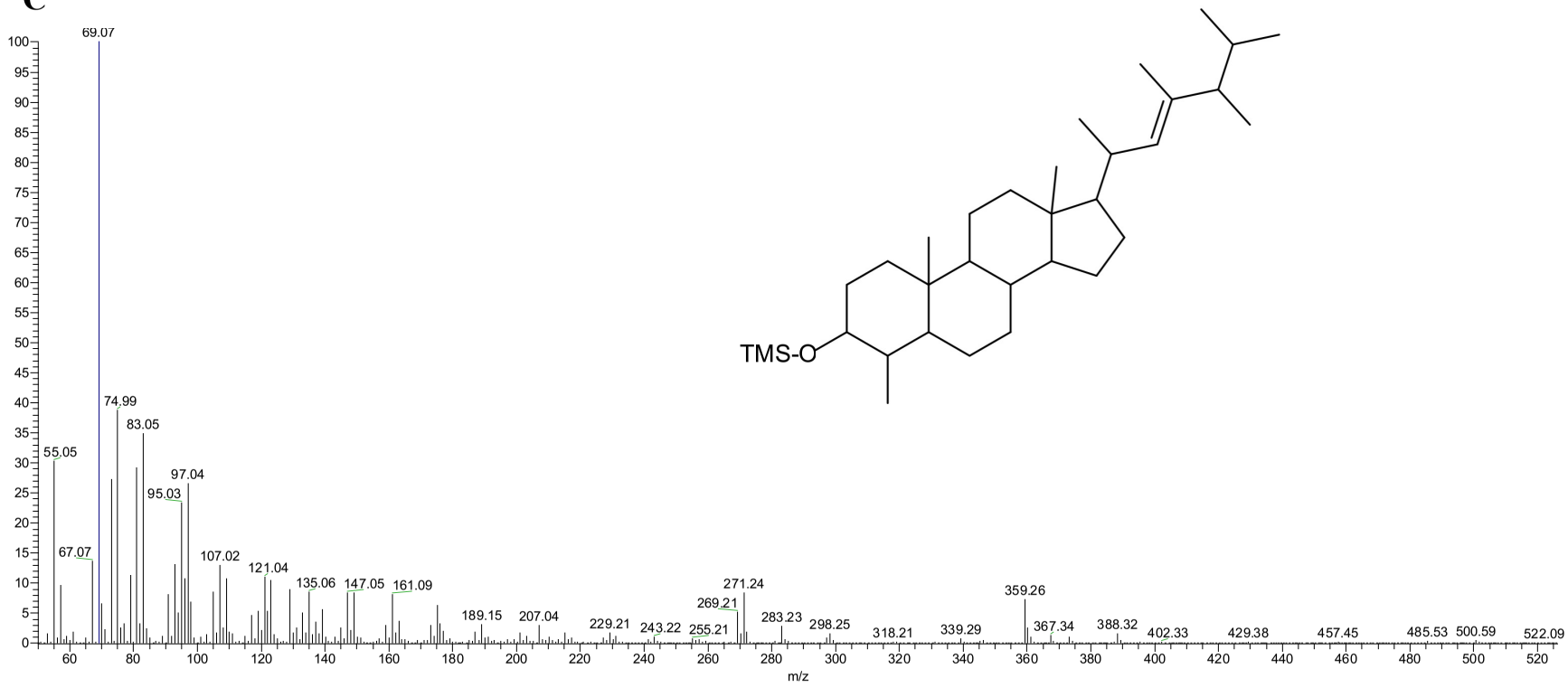

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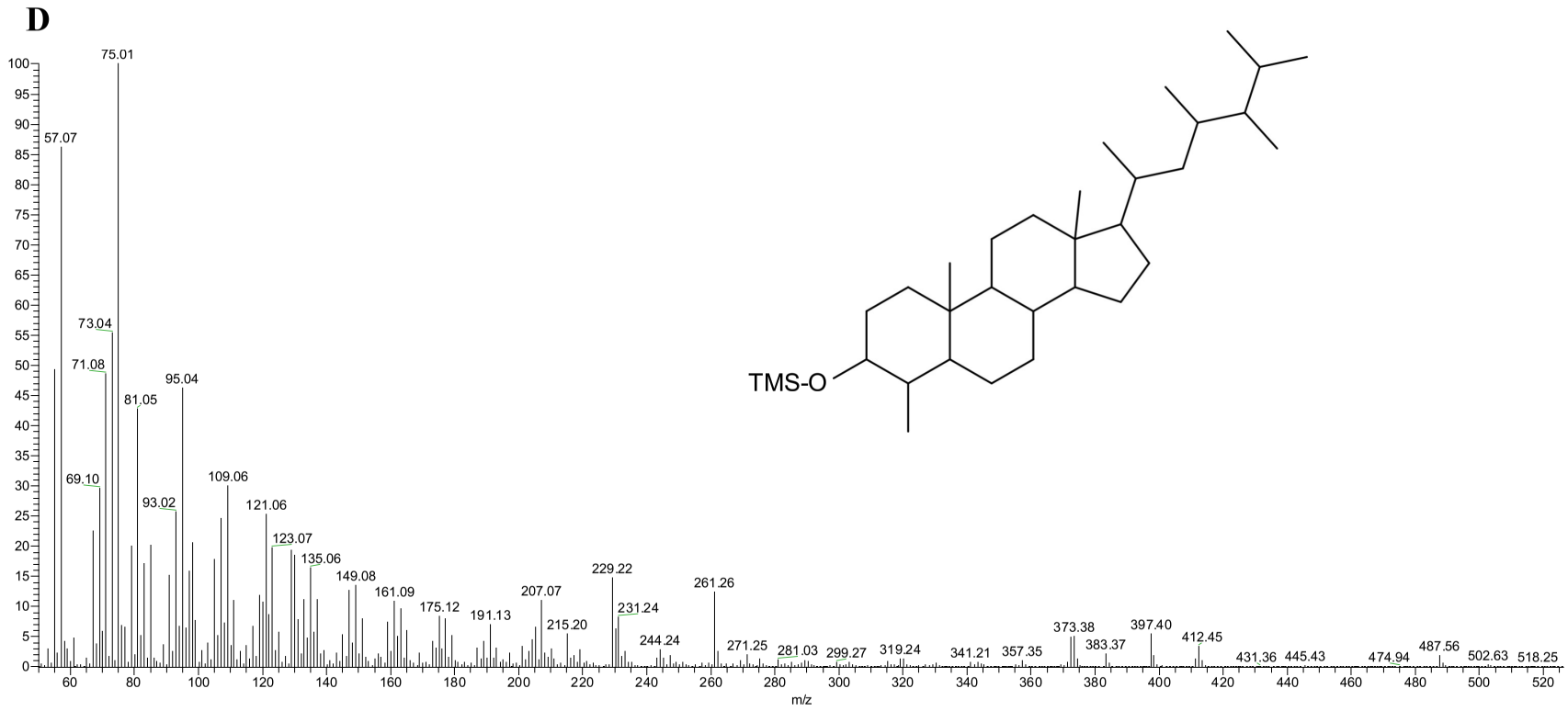
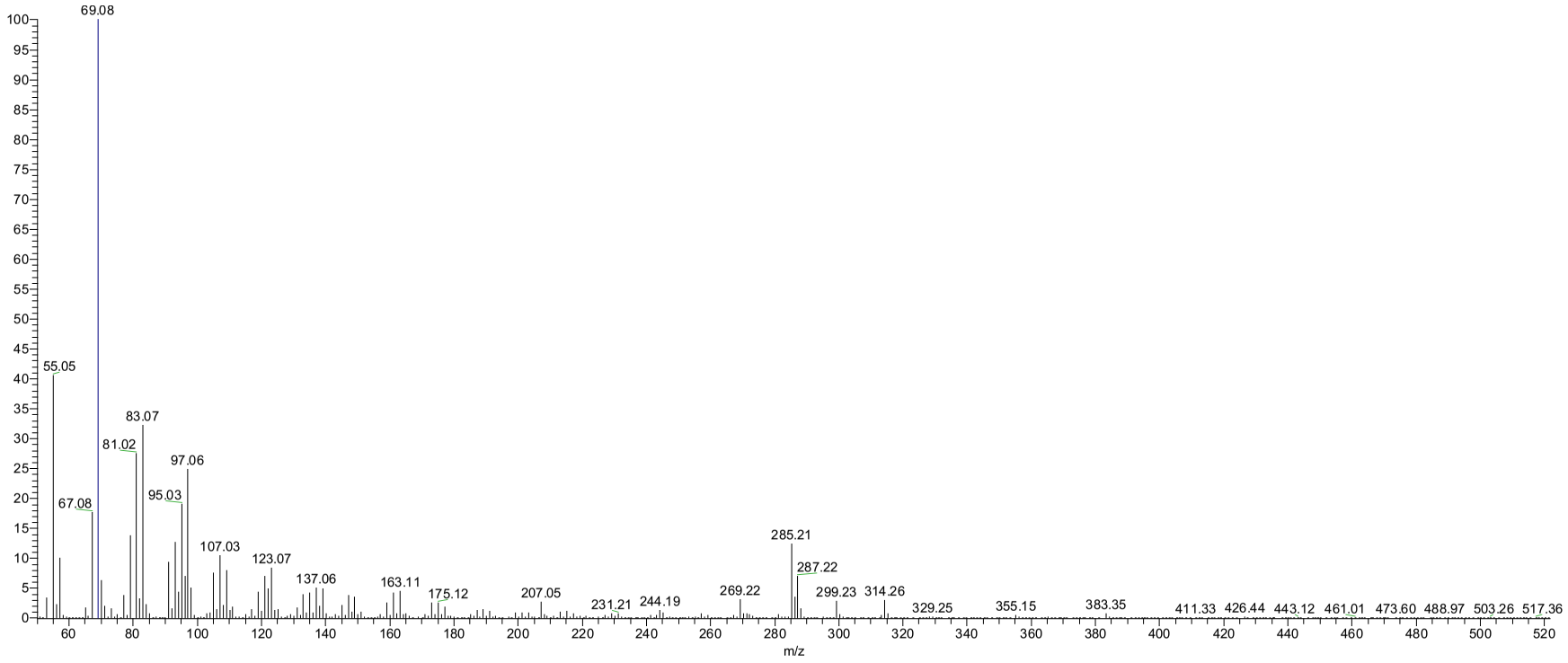


Figure 1. Major sterols found in the free sterol fraction of *Zooxanthella nutricula* CCMP 3427. **(A)** GC/MS spectrum of cholesta-5,22E-dien-3β-ol (22-dehydrocholesterol) as its TMS derivative (*m/z* 456) from the free sterol fraction of *Z. nutricula* alongside the corresponding chemical structure and **(B)** the GC/MS spectrum of an authentic standard cholesta-5,22Z-dien-3β-ol (22-dehydrocholesterol) as its TMS derivative (*m/z* 456). **(C)** GC/MS spectrum of 4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol (dinosterol) as its TMS derivative (*m/z* 500) from the free sterol fraction of *Z. nutricula* alongside the corresponding chemical structure. **(D)** GC/MS spectrum of 4α,23,24-trimethyl-5α-cholestan-3β-ol (dinostanol) as its TMS derivative (*m/z* 502) from the free sterol fraction of *Z. nutricula* alongside the corresponding structure.

A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443.12
461.01
473.60
488.97
503.26
517.36
m/z

(*Figure 2 Continued*)

B

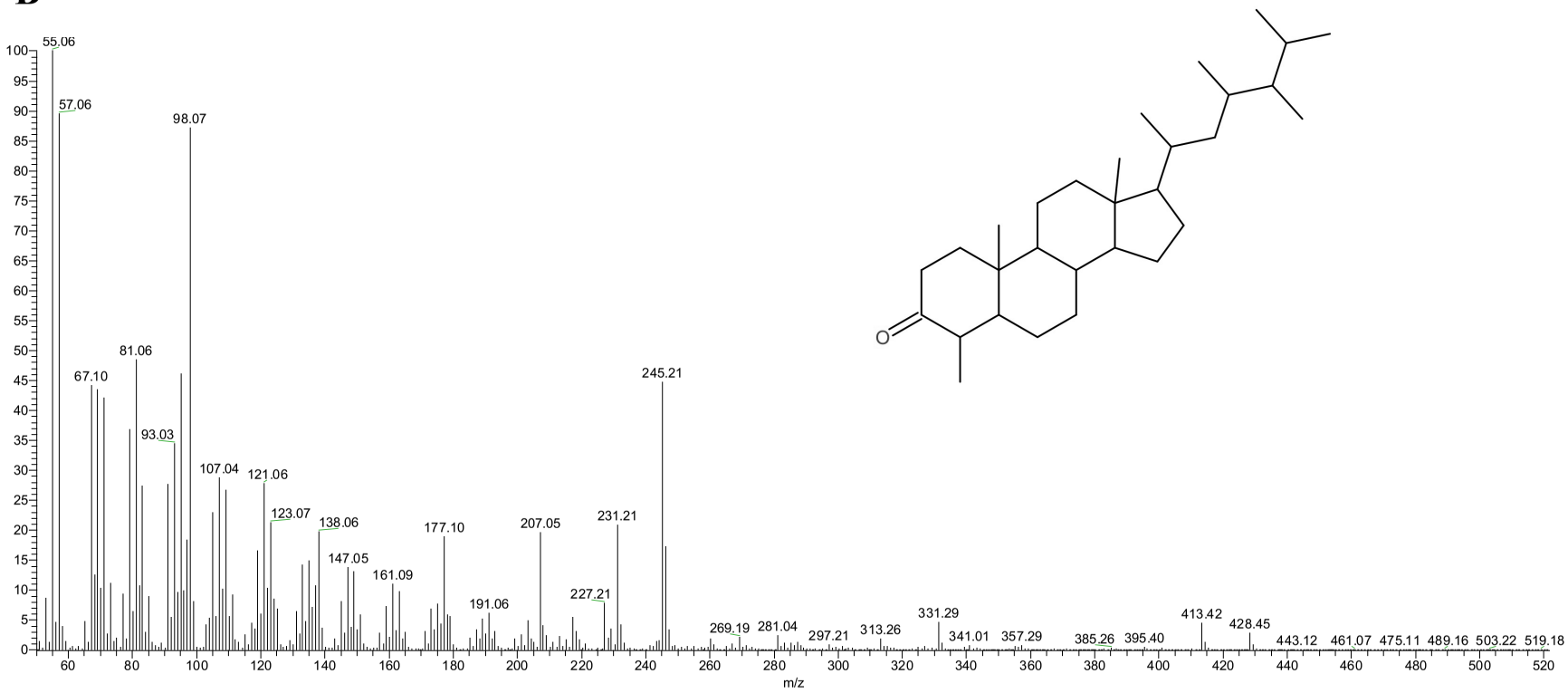
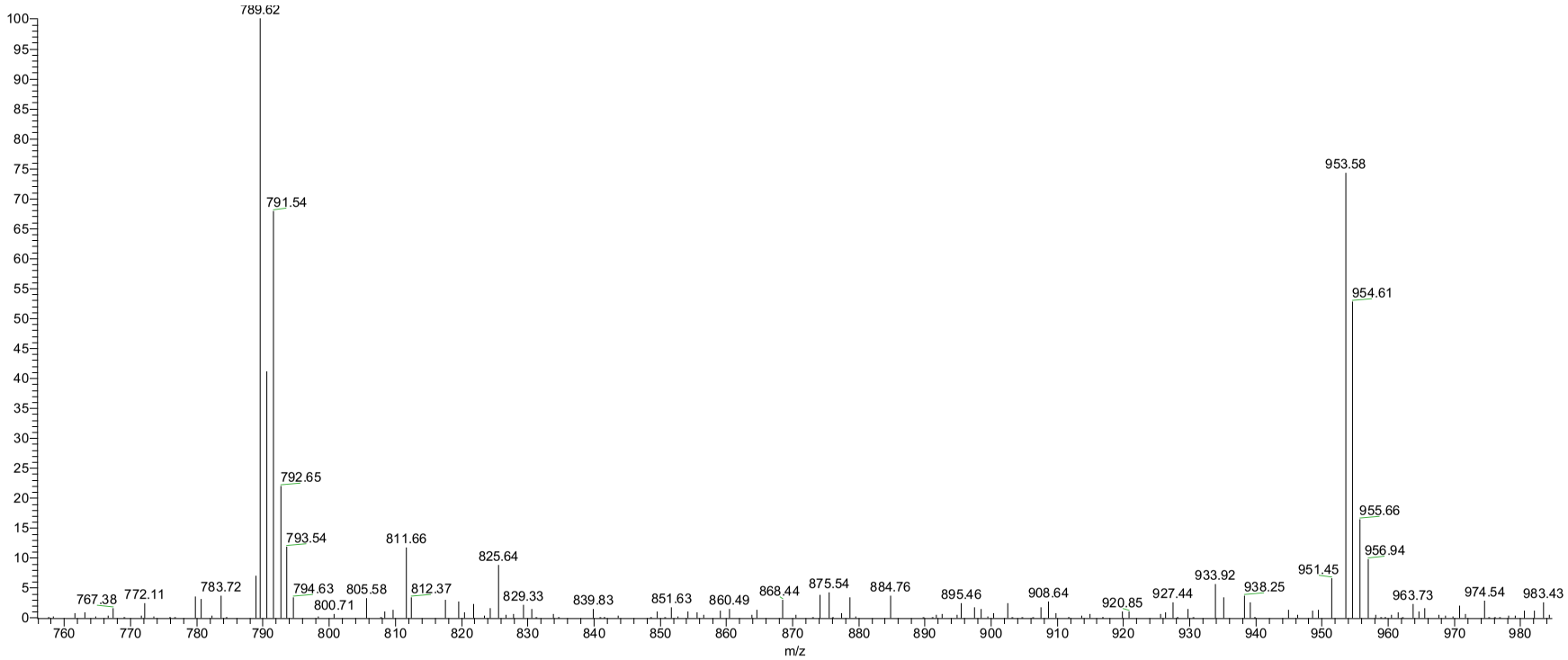
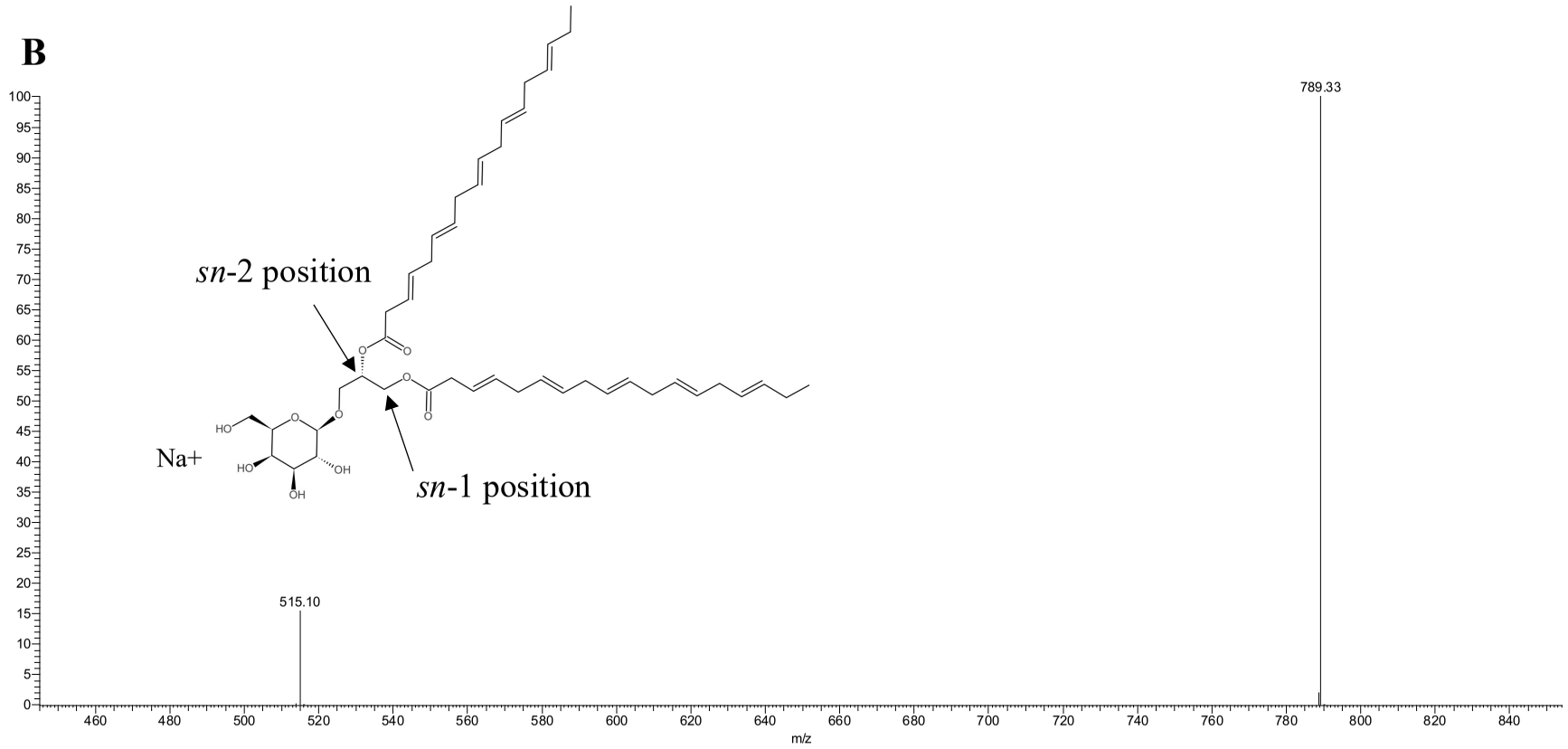


Figure 2. The major sterol and steroidal ketone found in the sterol ester fraction of *Zooxanthella nutricula* CCMP 3427. **(A)** GC/MS spectrum of unidentified $C_{29:1}$ sterol as its TMS derivative (*m/z* 488) from the sterol ester fraction of *Z. nutricula*. **(B)** GC/MS spectrum of the steroidal ketone 4α,23,24-trimethyl-5a-cholestan-3-one (dinostanone, *m/z* 428) from the sterol ester fraction of *Z. nutricula* alongside the corresponding chemical structure.

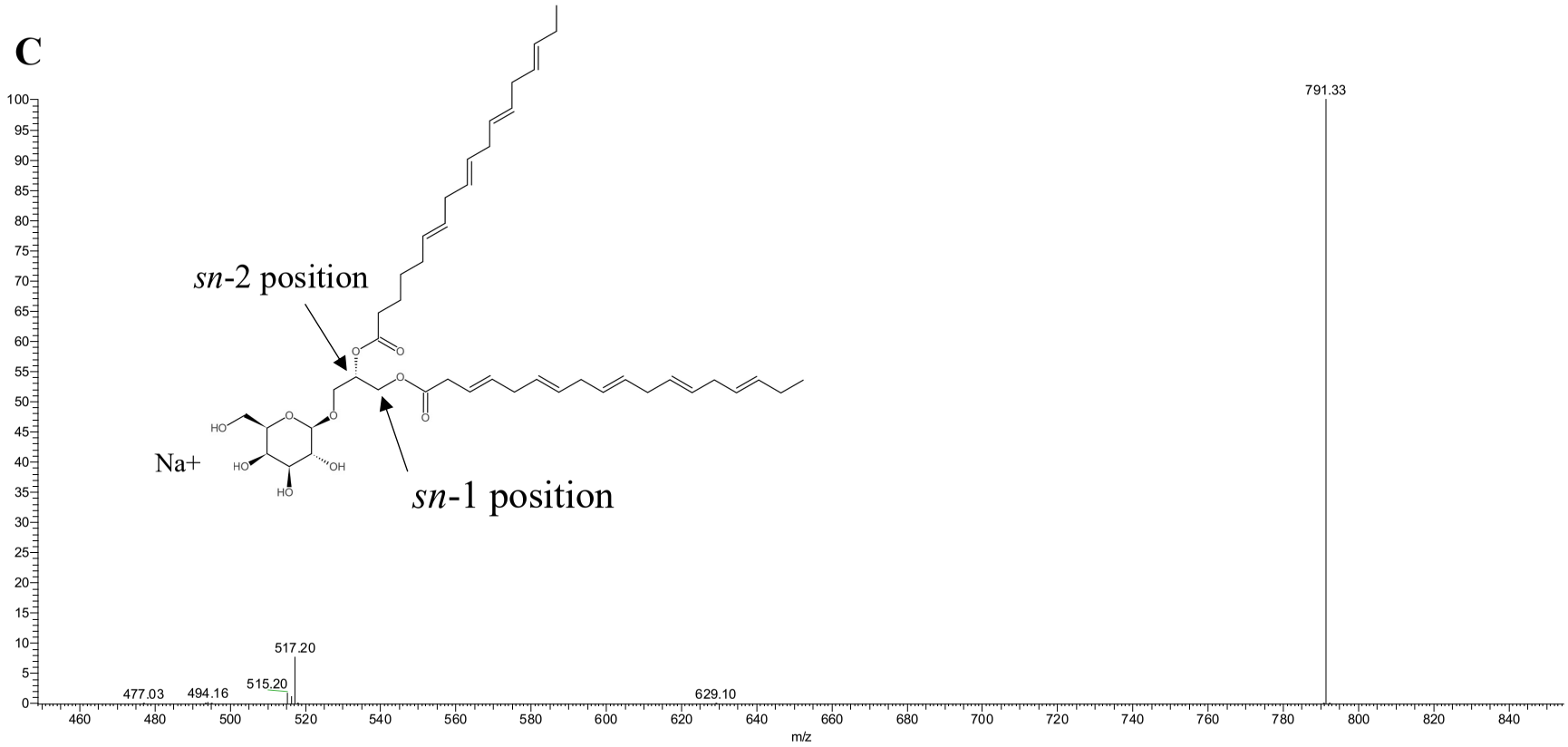
A


789.62
791.54
792.65
793.54
794.63
800.71
805.58
811.66
812.37
825.64
829.33
839.83
851.63
860.49
868.44
875.54
884.76
895.46
908.64
920.85
927.44
933.92
938.25
951.45
953.58
954.61
955.66
956.94
963.73
974.54
983.43
767.38
772.11
783.72
m/z

(*Figure 3 Continued*)



(*Figure 3 Continued*)



(*Figure 3 Continued*)

D

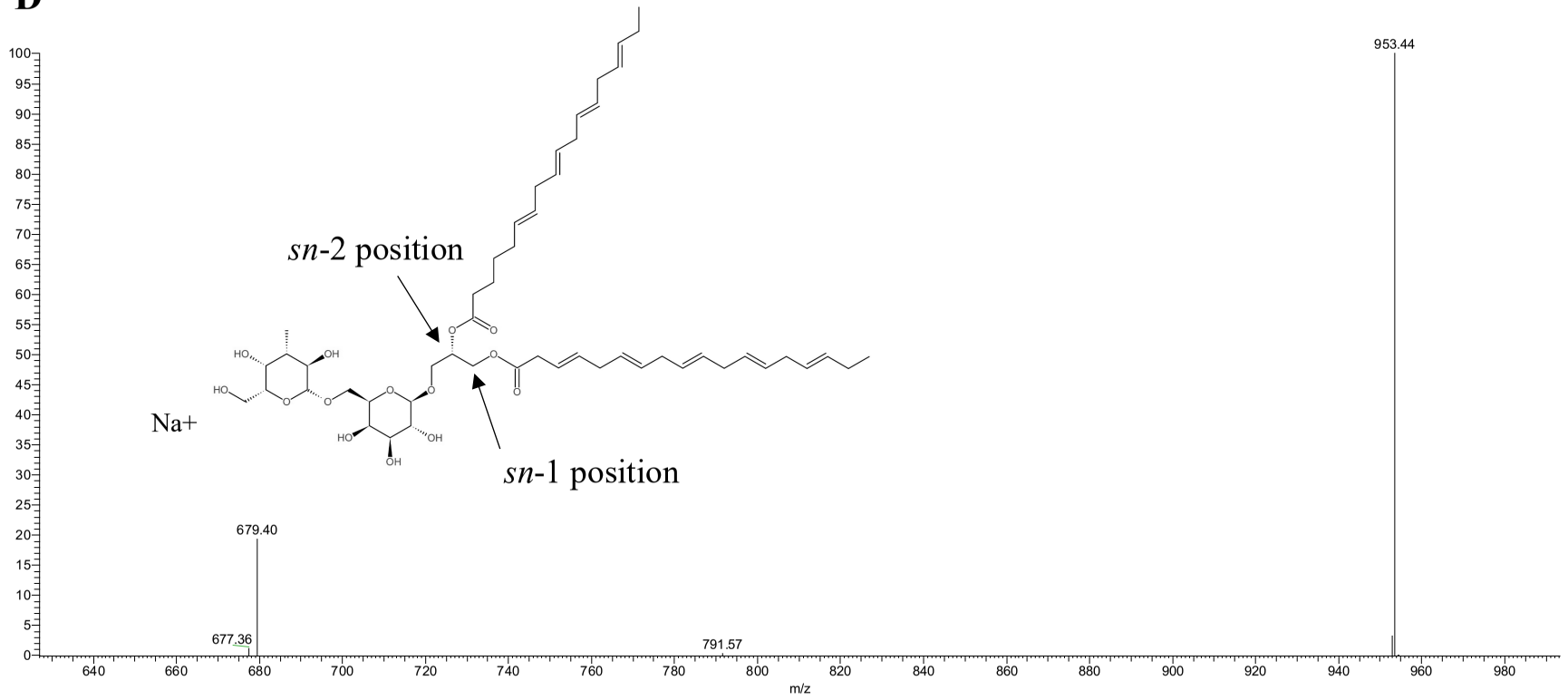


Figure 3. The galactolipids found in *Zooxanthella nutricula* CCMP 3427. **(A)** Full-scan ESI/MS spectrum of galactolipids from *Z. nutricula*. Galactolipids as Na^+ adducts are 18:5/18:5 MGDG (*m/z* 789), 18:5/18:4 MGDG (*m/z* 791), and 18:5/18:4 DGDG (*m/z* 953). **(B)** ESI/MS/MS spectrum of 18:5/18:5 MGDG (as its Na^+ adduct) from *Z. nutricula*. The ion at *m/z* 789 represents the mass of the intact galactolipid. The ion at *m/z* 515 represents the mass of the galactolipid after cleavage of the 18:5 fatty acid from either the *sn*-1 or the *sn*-2 position as depicted in the corresponding chemical structure. **(C)** ESI/MS/MS spectrum of 18:5/18:4 MGDG (as its Na^+ adduct) from *Z. nutricula*. The ion at *m/z* 791 represents the mass of the intact galactolipid. The ion at *m/z* 517 represents the mass of the galactolipid after preferential cleavage of the 18:5 fatty acid from the *sn*-1 position. The ion at *m/z* 515 represents the mass of the galactolipid after less preferential cleavage of the 18:4 fatty acid from the *sn*-2 position as depicted in the corresponding chemical structure. **(D)** ESI/MS/MS spectrum of 18:5/18:4 DGDG (as its Na^+ adduct) from *Z. nutricula*. The ion at *m/z* 953 represents the mass of the intact galactolipid. The ion at *m/z* 791 represents the mass of the galactolipid after cleavage of one galactose. The ion at *m/z* 679 represents the mass of the galactolipid after preferential cleavage of the 18:5 fatty acid from the *sn*-1 position. The ion at *m/z 677* represents the mass of the galactolipid after less preferential cleavage of the 18:4 fatty acid from the *sn*-2 position as depicted in the corresponding chemical structure.