TAKING THE HEAT:

THE EFFECT OF GROWTH TEMPERATURE ON LIPIDS OF THE DIATOMS

by

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I dedicate this research to my wife. I love you very much, Kristen.

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I would like to thank my wife Kristen and both of our families for their unwavering love and support throughout this long process. Without Kristen's great sacrifices, I would not have been able to fulfill this dream of mine. Without her love and support I probably would have gone crazy a long time ago. I would also like to thank Dr. Jeffrey Leblond for persevering with me and pushing me to do great work. I have to thank Dr. Jeremy Dahmen and Dr. Jean-Luc Mouget for their help and support in getting these manuscripts published. Finally, I would like to thank my friends, colleagues, and professors at MTSU for being there for me when I needed them.

ABSTRACT

Diatoms are one of the largest groups of primary producers in the oceans, and because of their environmental and increasing economic importance, more information is being revealed about their lipid biochemistry. Mass spectrometry techniques were used to examine the lipids of diatoms, with particular focus on how growth temperature would affect different lipid classes. Galactolipid-associated fatty acid composition and regiochemistry was examined in a selection of diatoms. Two centric diatoms, *Skeletonema marinoi* and *Thalassiosira weissflogii*, and one pennate diatom, *Phaeodactylum tricornutum*, contained primarily C_{20}/C_{16} (*sn*-1/*sn*-2) and C_{18}/C_{16} forms of the galactolipids. The other pennate diatoms, *Haslea ostrearia* and *Navicula perminuta*, contained primarily C_{18}/C_{16} or C_{18}/C_{18} forms of these lipids. This shows a split in galactolipid profiles that does not correspond with the phylogeny of these organisms and their plastids.

The effect of different growth temperatures on the galactolipid fatty acids of *H. ostrearia* and *P. tricornutum* was also examined. At 20°C, *H. ostrearia* and *P. tricornutum* were rich in eicosapentaenoic acid (EPA; $C_{20:5}$) at the *sn*-1 position and C_{16} fatty acids at the *sn*-2 position of the galactolipids. At 30°C, however, *H. ostrearia* and *P. tricornutum* contained no EPA or other C_{20} fatty acids, but rather contained higher percentages of C_{18} fatty acids at *sn*-1. When exposed to a higher growth temperature, both species cope by eliminating the longest and shortest fatty acid chains, as well as

decreasing the total number of unsaturations, possibly reflecting a difference in their autecologies.

Finally, the hydrocarbons produced by *P. tricornutum* at 20° and 30°C were examined. Innate production of hydrocarbons in diatoms could eventually lead to these organisms being used as direct sources of biofuels. *P. tricornutum* produced a number of different hydrocarbons, namely octane, undecane, nonadecane, and heneicosane at 20°C. At 30°C, however, the alkanes produced were heptadecane, octadecane, nonadecane, and eicosane, as well as three alkenes: heptadecene, octadecene, and nonadecene. The hydrocarbons produced are indeed in the ranges of hydrocarbons seen in petroleum fuels, like gasoline and diesel, and temperature does have a large impact on the hydrocarbons are produced.

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INTRODUCTION

BIOLOGY OF DIATOMS

Diatoms are among the most common types of phytoplankton with at least 100,000 species distributed among over 1,250 genera in a wide range of habitats, but with most living pelagically in freshwater or seawater (Seckbach & Kociolek, 2011). Among algae, diatoms are distinctive by being encased in silica cell walls called frustules (Hamm et al., 2003). In fact, the name diatom references that this frustule is often symmetrical. This symmetry separates the diatoms into two classes: radially symmetrical (centric) and bilaterally symmetrical (pennate) diatoms. Centric and pennate diatoms are found ubiquitously in both freshwater and marine environments. The pennate diatoms are the younger of the two groups, but are also the most diverse, and contain numerous features, besides their symmetry, that distinguish them from the centrics (Bowler et al., 2008).

Diatoms, of the phylum Heterokontophyta and a member of the supergroup Chromalveolata, are one of the primary producers in marine ecosystems, and indeed the planet (Seckbach & Kociolek, 2011). Marine diatoms often depict what is called "bloom and bust" behavior. Under favorable conditions, such as appropriate nutrients, temperature, and light values that are specific to a given species, diatoms are able to quickly reproduce, the "bloom", and dominate phytoplankton populations. Once the conditions become unfavorable, such as when the nutrients are depleted, the cells will die off and sink to deeper waters, the "bust", until the surviving cells are moved into the

upper layers again through vertical mixing (Furnas, 1990; Hecky & Kilham, 1988). Diatom blooms can also have negative environmental effects, as in the case of the toxin-producing diatom *Pseudo-nitzschia* off of the California coast which is responsible for a large number of sea lion strandings (Bargu et al., 2012).

Due to their ecological importance, diatoms have become the subject of many studies at the molecular level to understand their ability to dominate phytoplankton populations. One such area of study which focuses on the diatoms has been the origin of the chromalveolate chloroplast, which has been previously explained via the chromalveolate hypothesis, stating that all chromalveolate chloroplasts, which includes those of the diatoms, can be traced back to a single red algal ancestor (Cavalier-Smith, 1999); however, the monophyly of this group is currently in contention and is undergoing further study (Bodył et al., 2009; Green, 2011). The combination of two ancient endosymbiosis events, as well as supplemental horizontal gene transfer, has provided the chromalveolates with the genetic potential to become one of the largest groups of primary producers on the planet (Moustafa et al., 2009). At some point the members of this group, which also includes the cryptophytes, haptophytes, and alveolates as well as the heterokontophytes, acquired their plastid through secondary endosymbiosis of a red alga, explained by the presence of four membranes and chlorophyll *c* (Keeling, 2009). In recent studies of diatom genomes, however, there have been discoveries of nuclear genes of a green algal origin that are not the result of lateral gene transfer, suggesting a cryptic

endosymbiont likely related to prasinophyte-like green algae in an ancestor of chromalveolates (Kim & Graham, 2008).

SIGNIFICANCE OF GALACTOLIPIDS

As reviewed by Wada and Murata (2010), the photosynthetic machinery (the light-harvesting complexes and photosystems that carry out the light reactions of photosynthesis) is housed within a membrane that contains primarily the galactolipids mono- and digalactosyldiacylglycerol (MGDG and DGDG, respectively), with smaller amounts of the sulfolipid, sulfoquinovosyldiacylglycerol (SQDG), and the phospholipid, phosphatidylglycerol (PG). The galactolipids MGDG and DGDG have been shown to comprise around 75% of the total lipids of the leaves of higher plants, far outnumbering the phospholipids (Dörmann & Benning, 2002). These lipids have been shown to be closely integrated with the photosynthetic machinery of the chloroplasts, and as such they are vital to the photosynthetic processes. For example, MGDG and PG were shown to be closely positioned to, and even hydrogen bonded to, cyanobacterial photosystem I, while DGDG and PG were found in the light harvesting complex II of photosystem II (Dörmann & Benning, 2002; Mizusawa & Wada, 2012).

Despite their ecological importance there are few studies on diatom plastid lipid biochemistry. Yan et al. (2011) have previously examined the relative abundances of MGDG and DGDG, as well as the fatty acid regiochemistry, of the centric diatom genus,

Skeletonema. They found that this genus contained mostly MGDG and DGDG with eicosapentaenoic acid (EPA, $C_{20:5}$) at the *sn*-1 position while possessing mostly $C_{16:1}$, $C_{16:2}$, and $C_{16:3}$ fatty acids in the *sn*-2 position. Yongmanitchai and Ward (1993) analyzed the pennate diatom *Phaeodactylum tricornutum,* and found it likewise predominately contained MGDG and DGDG with EPA at the *sn*-1 position and $C_{16:1}$, $C_{16:3}$, and $C_{16:4}$ fatty acids at the *sn*-2 position. Dunstan et al. (1994) previously examined fatty acid composition of 14 diatom species, including representatives from the genera *Skeletonema, Thalassiosira, Navicula,* as well as *Haslea ostrearia*. Their study found that the diatoms collectively contained significantly large amounts of $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, and $C_{20:5}$ fatty acids.

In all photosynthetic organisms (cyanobacteria, algae, plants), fatty acid composition is important for stabilizing the membrane proteins associated with photosynthesis, such as the light-harvesting complexes and photosystems (Dörmann & Benning, 2002; Mironov et al., 2012; Mizusawa & Wada, 2012) and membrane fluidity is generally involved in the responses to temperature changes in the environment (Mikami & Murata, 2003; Los & Murata, 2004). Studies relating to the modulation of the membrane fatty acids as a function of temperature have been performed on different photosynthetic microorganisms, such as the chlorophyte *Dunaliella salina* (Lynch & Thompson, 1982; 1984), the dinoflagellate *Gyrodinium aureolum* (Parrish et al., 1993), and the haptophyte *Pavlova lutheri* (Tatsuzawa & Takizawa, 1995). For instance, an

increase in polyunsaturated fatty acids (PUFAs), a decrease in MGDG and almost no changes in DGDG were observed in *P. lutheri* grown at 15°C versus 25°C (Tatsuzawa & Takizawa, 1995). Specific studies dealing with the effect of temperature changes on the membrane fatty acid profile of diatoms in particular are lacking, and understanding the lipid biochemistry, with an emphasis on fatty acid regiochemistry, of these photosynthetically important organisms may provide insights into specific lipid-based survival strategies during changing environmental conditions, possibly giving an insight into their characteristic bloom or bust behavior.

SIGNIFICANCE OF HYDROCARBONS

Another prominent example of the increasing importance of diatoms in molecular biology is the continuing search for sustainable fuel sources and natural products for pharmacological use. Algae, such as the promising candidate *Botryococcus braunii*, are known to produce oils that can be made into biofuel through a process known as hydrocracking (Hillen et al., 2004; Metzer & Largo, 2005). Current prospects in the search for biofuels, such as ethanol and biodiesel, are lower in energy content and often incompatible with the petroleum-based fuel infrastructure (processing systems, engines based on gasoline/diesel, etc.) in which we live (Lee et al., 2008). To counteract this, the search is on for an organism that either produces naturally, or can be engineered to

produce, a fuel source that is both comparable in energy content and compatible to the current petroleum-based infrastructure (Ramachandra et al., 2009; Radakovits et al., 2010).

Diatoms are known to produce high amounts of lipids, anywhere from 50%-60% of their biomass weight, with about 75% of those lipids being neutral lipids such as triglycerides (TAGs), isoprenoids, and hydrocarbons (Ramachandra et al., 2009). The diatoms *Rhizoselenia setigera, Pleurosigma intermedium,* and *H. ostrearia*, as well as other members of the *Haslea* genus, have been found to produce a number of highly branched isoprenoid (HBI) isomers, some of which have been found to possess biological activity against tumors (Rowland et al., 2001). These very same HBIs can also be further processed to create biofuels, or have their biosynthetic pathways exploited to produce fuel substitutes such as isobutanol (Rowland et al., 2001; Lee et al., 2008). The biological purpose of these HBIs is currently unknown, although it has been shown that the unsaturation of the HBIs increases with increasing temperature, contrary to what would normally be expected during homeoviscous adaptation of membrane lipids like phospholipids and galactolipids, and they have been primarily examined for their possible pharmacological applications and presence in sediments as biomarkers of past diatom blooms (Rowland & Robson, 1990; Rowland et al. 2001). Smaller isoprenoids, such as the C_{15} isoprenoids farnesane and bisabolane, have the potential to become a source of biofuel, as their branches, double bonds, and ring structures satisfy the need for

molecules with fluidity at freezing cold as isoprenoid-derived biofuels are being considered for use as jet fuels; however, these same features also cause a decrease in combustion quality (Lee et al., 2008; Peralta-Yahya et al., 2012). Lipid accumulation, namely accumulation of triacylglycerols, has been studied in *P. tricornutum* under a number of stressful growth conditions, such as nitrogen depletion, and it has been a model for lipid metabolism studies as well (Hu et al., 2008; Valenzuela et al., 2012). As of this writing, no studies of short- to medium-chain hydrocarbon production in *P. tricornutum* have been performed.

EXPERIMENTAL PURPOSE

Contained in this manuscript are three different studies on the lipids of diatoms. The overarching goal for these studies was to collect information about the effects of temperature on the different classes of lipids. The galactolipids are a primary component of chloroplast membranes, and hydrocarbons possess a unique importance in that they are among a number of natural products being investigated for both pharmacological activity and the potential use as a biofuel source. The overall purpose of all three studies was to use of mass spectrometry to examine lipids in diatoms, and to determine the effect temperature has on certain lipid classes.

SUMMARY OF FINDINGS

In Chapter I, the galactolipids of five diatom species, two centric diatoms and three pennate diatoms, including the "blue" diatom *H. ostrearia* were profiled. The two centric diatoms, *Skeletonema marinoi* and *Thlassiosira weissflogii*, and the pennate diatom, *P. tricornutum*, contained primarily C_{20}/C_{16} (*sn*-1/*sn*-2) and C_{18}/C_{16} forms of MGDG and DGDG. The other pennate diatoms, *H. ostrearia* and *Navicula perminuta*, contained primarily C_{18}/C_{16} or C_{18}/C_{18} forms of MGDG and DGDG, indicating a previously unrecognized fatty acid diversity in diatom MGDG and DGDG.

In Chapter II, *P. tricornutum* and *H. ostrearia* were grown in triplicate at the same temperature as in the first experiment, 20°C, and a second set of triplicate cultures was grown at a higher temperature, 30°C, to determine the effects of temperature on their galactolipid profiles. At 20°C, *H. ostrearia* and *P. tricornutum* were rich in eicosapentaenoic acid (EPA; $C_{20:5}$) at the *sn*-1 position and in C_{16} fatty acids at the *sn*-2 position of MGDG and DGDG. At 30°C, however, *H. ostrearia* and *P. tricornutum* contained no EPA or other C_{20} fatty acids, but rather contained higher percentages of C_{18} fatty acids at *sn*-1. At 30°C, no galactolipid in either diatom contained more than 3 unsaturations on any of its fatty acids. Although these two species differed in galactolipid composition, they both possessed a similar method of acclimating their

galactolipids to a higher growth temperature: eliminating the longest and shortest fatty acid chains, as well as decreasing the total number of unsaturations.

In Chapter III, *P.tricornutum*'s ability to produce any notable hydrocarbons was examined, particularly short- to medium-chain (from C_4 to C_{23}) hydrocarbons which could have potential uses as biofuels, and the extent to which growth temperature had effect on the nature of the hydrocarbons produced. There was a notable difference in the hydrocarbon profile of *P. tricornutum* when grown at 30°C when compared to 20°C. A number of alkanes were detected at 20°C, ranging from eight carbons to twenty-one carbons. At 30°, however, the alkanes produced are very different with only one alkane appearing at both temperatures. Also at 30°C, three alkenes, or hydrocarbons containing a single double bond, were detected indicating that temperature does have some influence on hydrocarbon production.

CHAPTER I

MONO- AND DIGALACTOSYLDIACYLGLYCEROL COMPOSITION OF THE MARENNINE-PRODUCING DIATOM, *HASLEA OSTREARIA*: COMPARISON TO A SELECTION OF PENNATE AND CENTRIC DIATOMS

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INTRODUCTION

Diatoms are among the most common types of phytoplankton with at least 100,000 species distributed among over 1,250 genera in a wide range of habitats, but with most living pelagically in freshwater or seawater (Seckbach & Kociolek, 2011). Among algae, diatoms are distinctive by being encased in silica cell walls called frustules (Hamm et al., 2003). In fact, the name diatom references that this frustule is often symmetrical. This symmetry separates the diatoms into two classes: radially symmetrical (centric) and bilaterally symmetrical (pennate) diatoms. Centric and pennate diatoms are found ubiquitously in both freshwater and marine environments. The pennate diatoms are the younger of the two groups, but are also the most diverse, and contain numerous features, besides their symmetry, that distinguish them from the centrics (Bowler et al., 2008).

Due to their ecological importance as primary producers and bloom-formers, diatoms have become the subject of many studies at the molecular level to understand their ability to dominate phytoplankton populations. One such area of study has been the origin of the chromalveolate chloroplast, which has been previously explained via the chromalveolate hypothesis as all being derived from a single red algal ancestor (Cavalier-Smith, 1999); however, the monophyly of this group is currently in contention and is undergoing further study (Bodył et al., 2009; Green 2011). At some point the members of this group, which also includes the cryptophytes, haptophytes, and alveolates as well as the heterokontophytes, acquired their plastid through secondary endosymbiosis of a red alga, explained by the presence of four membranes, the outer one being continuous with the rough endoplasmic reticulum (RER) and the outer membrane of the nucleus (Keeling, 2009). In recent studies of diatom genomes, however, there have been

discoveries of nuclear genes of a green algal origin that are not the result of lateral gene transfer, suggesting a cryptic endosymbiont likely related to prasinophyte-like green algae in an ancestor of chromalveolates (Kim & Graham, 2008). The combination of two ancient endosymbiosis events, as well as supplemental horizontal gene transfer, has provided the chromalveolates with the genetic potential to become one of the largest groups of primary producers on the planet (Moustafa et al., 2009).

Most diatoms produce a number of photosynthetic pigments, such as chlorophylls *a* and *c* (green), beta-carotene (yellow), diadinoxanthin, diatoxanthin, and fucoxanthin (brown), that give them a green to golden-brown color (Bertrand et al., 2010). The "blue" pennate diatom, *Haslea ostrearia*, is notable in that it synthesizes and releases into its environment the water-soluble, polyphenolic, non-photosynthetic blue pigment marennine (Nassiri et al., 1998). *H. ostrearia* has been known to out-compete other algae in some oyster ponds in the Atlantic coast of France and release marennine. This release of marennine during the bloom into the water ponds not only "greens" the ponds, but the oysters filter the marennine out of the water and turn green themselves, becoming an expensive yet festive delicacy in some areas of Europe (Gastineau et al., 2012).

As reviewed by Wada and Murata (2010), the photosynthetic machinery, the light-harvesting complexes and photosystems that carry out the light reactions of photosynthesis, is housed within a membrane that contains primarily the galactolipids mono- and digalactosyldiacylglycerol (MGDG and DGDG, respectively), with smaller amounts of the sulfolipid, sulfoquinovosyldiacylglycerol (SQDG), and the phospholipid, phosphatidylglycerol (PG). The galactolipids MGDG and DGDG have been shown to comprise around 75% of the total lipids of the leaves of higher plants, far outnumbering

the phospholipids (Dörmann & Benning, 2002). These lipids have been shown to be closely integrated with the photosynthetic machinery of the chloroplasts, and as such they are vital to the photosynthetic processes. For example, MGDG and PG were shown to be closely positioned to, and even hydrogen bonded to, cyanobacterial photosystem I, while DGDG and PG were found in the light harvesting complex II of photosystem II (Dörmann & Benning, 2002; Mizusawa & Wada, 2012).

Despite their ecological importance there are few studies on diatom plastid lipid biochemistry. Yan et al. (2011) have previously examined the relative abundances of MGDG and DGDG, as well as the fatty acid regiochemistry, of the centric diatom genus, *Skeletonema*. They found that this genus contained mostly MGDG and DGDG with eicosapentaenoic acid (EPA, $C_{20:5}$) at the *sn*-1 position while possessing mostly $C_{16:1}$, $C_{16:2}$, and $C_{16:3}$ fatty acids in the *sn*-2 position. Yongmanitchai and Ward (1993) analyzed the pennate diatom *Phaeodactylum tricornutum*, and found it likewise predominately contained MGDG and DGDG with EPA at the *sn*-1 position and $C_{16:1}$, $C_{16:3}$, and $C_{16:4}$ fatty acids at the *sn*-2 position. Dunstan et al. (1994) previously examined fatty acid composition of 14 diatom species, including representatives from the genera *Skeletonema, Thalassiosira, Navicula,* as well as *H. ostrearia*. Their study found that the diatoms collectively contained significantly large amounts of $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, and $C_{20:5}$ fatty acids.

The objective of this study was to examine the MGDG and DGDG composition of *H. ostrearia*, and to compare it to a selection of centric and pennate diatoms. Examining the regiochemistry of these diatoms may give insight into the evolutionary history of the diatom plastid as well as the biosynthetic pathways utilized by diatoms to

produce these photosynthetically important and ubiquitous lipids. In this work, I have utilized positive-ion electrospray ionization/mass spectrometry (ESI/MS) and electrospray ionization/mass spectrometry/mass spectrometry (ESI/MS/MS) to describe the most abundant molecular forms, with regiochemical distribution of associated fatty acids, of MGDG and DGDG present in *H. ostrearia, P. tricornutum, Skeletonema marinoi* (renamed from *S. costatum*), *Navicula perminuta,* and *Thalassiosira weissflogii.*

MATERIALS AND METHODS

Cultures and growth conditions

Cultures of *Skeletonema marinoi* Sarno et Zingone CCMP 1332 (renamed from *Skeletonema costatum*), *Phaeodactylum tricornutum* Bohlin CCMP 1327, *Navicula perminuta_cf* Grunow CCMP 1305, and *Thalassiosira weissflogii* (Grun.) Fryxell et Hasle CCMP 1051 were obtained from the National Center for Marine Algae and Microbiota (NCMA, East Boothbay, Maine, USA). *Haslea ostrearia* (Gaillon) Simonsen NCC 344 was obtained from the Nantes Culture Collection (NCC, Université de Nantes, 2 Rue de La Houssiniere, 44322 Nantes, France). Each strain was grown in 2L of L1 medium at 20°C, an irradiance of ~50 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}, and a light:dark cycle of 14:10 hours; they were harvested according to the methodology of Leblond & Chapman (2000).

Lipid extraction and fractionation

Total lipids were extracted, and galactolipids separated from other lipid classes according to the techniques described by Leblond and Chapman (2000). Briefly, the total lipid extracts were separated into five component lipid fractions on columns of activated

Unisil silica (1.0 g, 100–200 mesh, activated at 120C; Clarkson Chromatography, South Williamsport, PA, USA). The following solvent regime was used to separate lipids according to polarity, with fraction 5 eluting the most polar lipids (Leblond & Chapman, 2000): (1) 12 mL methylene chloride (sterol esters), (2) 15 mL 5% acetone in methylene chloride with 0.05% acetic acid (free sterols, tri- and diacylglycerols, and free fatty acids), (3) 10 mL 20% acetone in methylene chloride (monoacylglycerols), (4) 45 mL acetone (chloroplast glycolipids, including MGDG, DGDG, and sulfoquinovosyldiacylglycerol [SQDG]), and (5) 15 mL methanol with 0.1% acetic acid (polar lipids, including phosphorous- and non-phosphorous-containing lipids).

Mass spectrometry of lipids

Following the procedure of Gray et al. (2009), the lipids of fraction 4 were suspended in methanol, chloroform, and 50 mM sodium acetate (Welti et al., 2002) prior to examination via mass spectrometry. The resulting MGDG and DGDG sodium adducts were analyzed using positive-ion ESI/MS full scans from 100 to 2,000 Da through direct injection (5 μL sample volume into a methylene chloride carrier solvent at 0.5 mL· min^{-1}) into a Finnigan DecaXP ion trap mass spectrometer (Waltham, MA, USA). The abundance of each lipid was determined by calculating relative percent composition based on peak height of raw data in the positive-ion ESI/MS full-scan mode. Subsequent positive-ion ESI/MS/MS was performed using collision energy between 37.5% and 48%, and major cleaved fatty acids were identified by the differences between the masses of the original ions and their fragments. Regiochemistry was determined by positive ESI /MS/MS according to the methodology of Guella et al. (2003).

RESULTS

The five diatom isolates seemingly formed two groups, with each having a different major form of MGDG, one with C_{20}/C_{16} carbon skeletons in its primary fatty acids, the other with C_{18}/C_{16} carbon skeletons (Table 1). Three of the diatoms, *S. marinoi*, *T. weissflogii*, and *P. tricornutum*, primarily contained forms of MGDG and DGDG with $C_{20:5}$ and $C_{16:3}$ as the most abundant fatty acids. Positive-ion ESI/MS/MS (see methodological description below) determined the regiochemical determinations (*sn*-1/*sn*-2) to be $C_{20:5}/C_{16:3}$ MGDG *(m/z* 793), the most abundant lipid in all three cultures. *P. tricornutum* contained three *m/z* 795 lipids which were found to be a $C_{20:5}/C_{16:2}$ form of MGDG and a lesser amounts of a $C_{20:4}/C_{16:3}$ form and a $C_{20:3}/C_{16:4}$ form. *P. tricornutum* also contained $C_{20:5}/C_{16:4}$ *(m/z* 791), and $C_{20:5}/C_{16:3}$ (*m/z* 793) forms of MGDG at approximately 15.4% and 19.5% relative abundance respectively, and a number of other forms of MGDG at less than 10% relative abundance. *P. tricornutum* also contained a number of forms of DGDG at relative abundances less than 10%, the highest of these being a $C_{16:1}/C_{16:1}$ form (*m/z* 911) at 6.8% and a $C_{16:1}/C_{16:0}$ (*m/z* 913) form at 6.1% relative abundance, and a $C_{20:5}/C_{16:1}$ (*m/z* 959) form at 5.5% relative abundance. *S. marinoi* contained two *m/z* 745 lipids, which were found to be a $C_{16:1}/C_{16:3}$ form at close to 10% relative abundance and a $C_{16:2}/C_{16:2}$ form of MGDG at a 2% relative abundance, and a $C_{20:5}/C_{16:1}$ (*m/z* 959) form of DGDG at approximately 30% relative abundance. *T. weissflogii* contained two other forms of MGDG with saturated fatty acids, $C_{14:0}/C_{16:1}$ (*m/z* 723) and $C_{16:0}/C_{16:3}$ (*m/z* 747), as well as two polyunsaturated forms, $C_{16:3}/C_{16:3}$ (*m/z* 741) and another *m/z* 747 lipid found to be $C_{16:1}/C_{16:2}$.

Table 1. Relative abundance (in % of total fragment height using listed masses) of galactolipids as determined via positive-ion ESI/MS

Galactolipid†	Mass‡	*Skeletonema marinoi* CCMP 1332	*Thalassiosira marinoi* CCMP 1052	*Phaeodactylum tricornutum* CCMP 1327	*Navicula perminuta* CCMP 1305	*Haslea ostrearia* NCC 344
14:0/16:3 MGDG	719			Tr		
14:0/16:1 MGDG	723		12.6	2.3		
16:3/16:3 MGDG	741		16.1			4.4
16:1/16:4 MGDG	743			2.5		
16:2/16:3 MGDG	743			1.0		
16:1/16:3 MGDG	745	9.8		3.3		
16:2/16:2 MGDG	745	2.0		Tr		
16:0/16:3 MGDG	747		6.1	Tr		1.3
16:1/16:2 MGDG	747		5.2	2.3		1.0
16:1/16:1 MGDG	749			3.6		
16:2/16:0 MGDG	749			Tr		
16:1/16:0 MGDG	751			5.2		3.9
18:4/16:3 MGDG	767					1.6
18:3/16:3 MGDG	769					30.6
18:3/16:2 MGDG	771					8.6
18:2/16:3 MGDG	771					4.4
18:4/16:0 MGDG	773				43.6	Tr
18:3/16:1 MGDG	773					2.3
18:2/16:2 MGDG	773					2.2
18:1/16:3 MGDG	773					Tr
18:1/16:1 MGDG	777			1.5		
18:2/16:0 MGDG	777				34.5	
20:5/16:4 MGDG	791			15.4		
20:5/16:3 MGDG	793	58.4	52.4	19.5	12.9	14.9
20:5/16:2 MGDG	795			6.1		4.2
20:4/16:3 MGDG	795			1.6		Tr
20:3/16:4 MGDG	795			Tr		
18:3/18:3 MGDG	797					5.6
20:5/16:1 MGDG	797			2.5		2.0
20:4/16:2 MGDG	797			Tr		
18:2/16:3 MGDG	799					1.3
20:5/16:0 MGDG	799			2.9		1.5
20:4/16:1 MGDG	799			Tr		
16:1/16:1 DGDG	911			6.8		
16:1/16:0 DGDG	913			6.1		
18:1/14:0 DGDG	913			Tr		
18:3/16:3 DGDG	931				9.0	2.8
18:3/16:0 DGDG	937					1.2
18:2/16:0 DGDG	939					1.4
18:1/16:1 DGDG	939			1.6		
20:5/16:2 DGDG	957		7.5	1.4		
18:3/18:3 DGDG	959					2.2
20:5/16:1 DGDG	959	29.8		5.5		Tr
20:5/16:0 DGDG	961			2.3		
20:4/16:1 DGDG	961			Tr		

†$[M + Na]^+$; notation represents number of carbons : number of double bonds (*sn*-1/*sn*-2).

‡Mass rounded down to nearest odd number for the purpose of simplification. Blank space, not detected. Tr, Trace amounts (less than 1%) were detected.

T. weissflogii also possessed a single $C_{20:5}/C_{16:2}$ form of DGDG (*m/z* 957) at 7.5% relative abundance. No C_{18} fatty acids were found as part of the major forms of MGDG and DGDG produced by *S. marinoi, T. weissflogii,* or *P. tricornutum*.

The remaining two diatoms, *H. ostrearia* and *N. perminuta*, possessed a variety of C_{18}/C_{16} MGDG forms (Table 1). *H. ostrearia* possessed $C_{18:3}/C_{16:3}$ (*m/z* 769, Fig. 1A) as its primary form of MGDG with 30.6% relative abundance along with lesser amounts of two *m/z* 771 lipids, found to be $C_{18:2}/C_{16:3}$ and $C_{18:3}/C_{16:2}$ forms of MGDG (Fig. 1B), and $C_{18:3}/C_{18:3}$ MGDG (*m/z* 797, Fig. 1C), and small amounts of other C_{16}/C_{16}, C_{18}/C_{16}, and high amounts of C_{20}/C_{16} forms of MGDG seen in Table 1. *H. ostrearia* also contained five forms of DGDG: $C_{18:3}/C_{16:3}$ (*m/z* 931, Fig. 1D), $C_{18:3}/C_{16:0}$ (*m/z* 937), $C_{18:2}/C_{16:0}$ (*m/z* 939), $C_{18:3}/C_{18:3}$ (*m/z* 959), and trace amounts of $C_{20:5}/C_{16:1}$ (*m/z* 959). *N. perminuta* possessed $C_{18:2}/C_{16:0}$ (*m/z* 777) and $C_{18:4}/C_{16:0}$ (*m/z* 773) forms of MGDG, at 34% and 44% relative abundance respectively, and 13% relative abundance of the $C_{20:5}/C_{16:3}$ (*m/z* 793) form of MGDG. *N. perminuta* also possessed a sole form of DGDG in $C_{18:3}/C_{16:3}$ (*m/z* 931) at 9% relative abundance.

In order to determine the regiochemistry of the fatty acids attached to these lipids, I performed positive-ion ESI/MS/MS. When our ion of interest from the full-scan data was subjected to ESI/MS/MS, the resulting spectrum was analyzed for peaks resulting from fragmentation of the intact lipid molecule. Subtracting the *m/z* of the peaks in the ESI/MS/MS spectra from the *m/z* of the intact molecule I was able to determine the mass of the molecule that was lost during fragmentation, the largest peak being

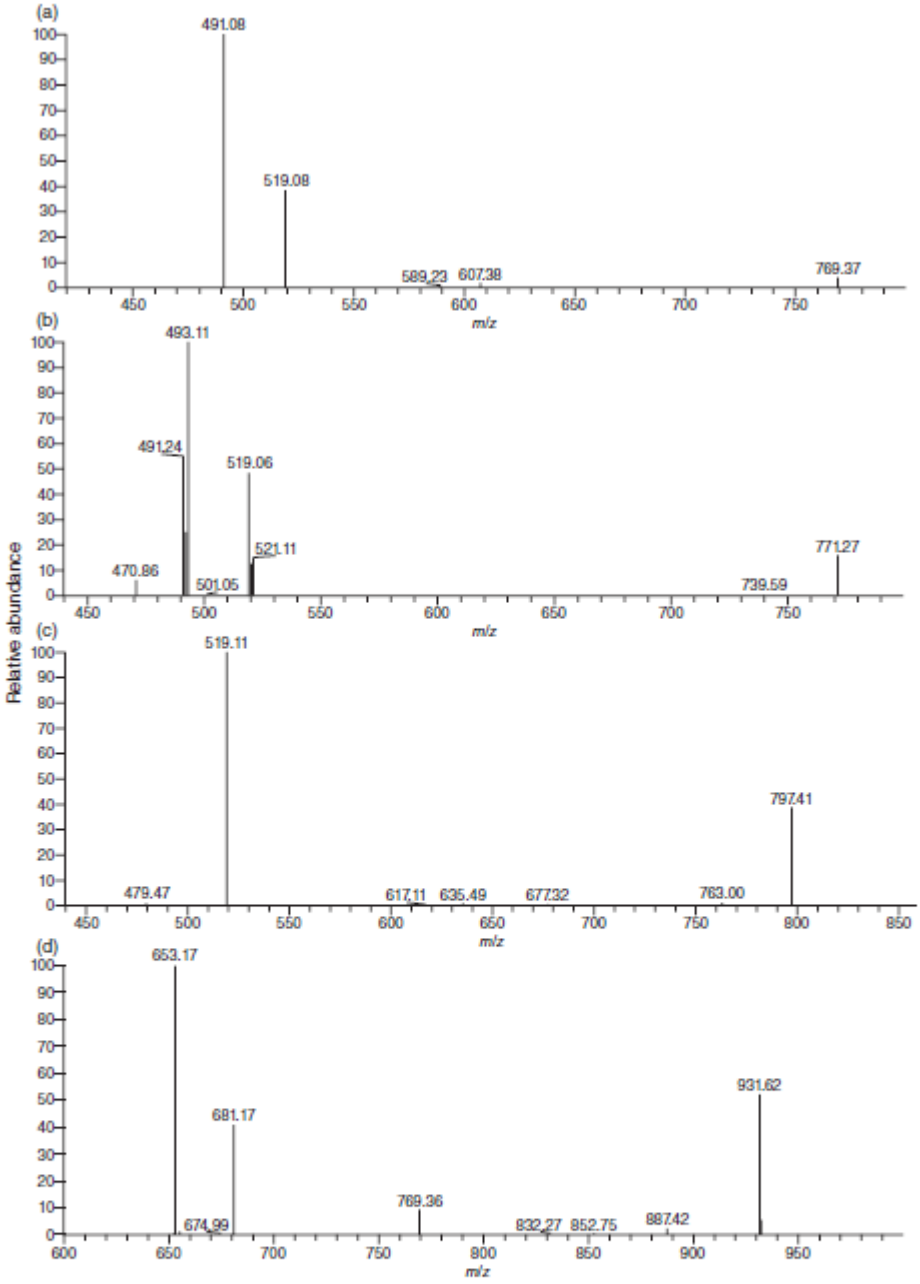

Fig. 1. Positive-ion electrospray ionization/mass spectrometry/mass spectrometry (ESI/MS/MS) spectra of four glycolipids from *Haslea ostrearia* NCC 344: (a) $C_{18:3}/C_{16:3}$ monogalactosyldiacylglycerol (MGDG) (molecular ion of *m/z* 769). The *m/z* 491 fragment represents the lipid minus $C_{18:3}$ from the *sn*-1 position, and the *m/z* 519 fragment represents the lipid minus $C_{16:3}$ from the *sn*-2 position; (b) A mixture of $C_{18:3}/C_{16:2}$ and $C_{18:3}/C_{16:3}$ MGDG molecules (molecular ion of *m/z* 771). The *m/z* 493 fragment represents the lipid minus $C_{18:3}$ from the *sn*-1 position, and the *m/z* 519 fragment represents the lipid minus $C_{16:2}$ from the *sn*-2 position. The *m/z* 491 fragment represents the second lipid minus $C_{18:2}$ from the *sn*-1 position, and the *m/z* 521 fragments represents the second lipid minus $C_{16:3}$ from the *sn*-2 position; (c) $C_{18:3}/C_{18:3}$ MGDG (molecular ion of *m/z* 797). The *m/z* 519 fragment represents the lipid minus $C_{18:3}$ from the *sn*-1 and *sn*-2 positions; (d) $C_{18:3}/C_{16:3}$ digalactosyldiacylglycerol (DGDG) (molecular ion of *m/z* 937). The *m/z* 653 fragment represents the lipid minus $C_{18:3}$ from the *sn*-1 position, and the *m/z* 681 fragment represents the lipid minus $C_{16:3}$ from the *sn*-2 position.

associated with the fatty acid attached to the *sn*-1 position on the glycerol backbone (Guella et al., 2003). For example, in Fig. 1A the *m/z* of the intact molecule was indicated by the small peak at *m/z* 769. The largest peak in Fig. 1A (*m/z* 491) was then subtracted from the intact molecule showing the cleavage of a fatty acid with a mass of 278, indicating a $C_{18:3}$ fatty acid. The mass of the second fatty acid was found likewise using the smaller peak on the spectra (*m/z* 519) and subtracting its mass from the intact molecule. This showed a cleavage of a fatty acid with mass 250, indicative of a $C_{16:3}$ fatty acid. The regiochemistry was assigned according to peak height, with the largest peak showing a preferential cleavage of the fatty acid from the *sn*-1 position while the smaller peak results from the cleavage of the fatty acid from the *sn*-2 position.

Those spectra which had more than two fragments, such as Fig. 1B, indicated a mix of two lipids with different fatty acids. The procedure is the same regarding the subtraction of the peaks from the intact molecule mass to find the mass of the cleaved fatty acids. Since the number of carbons and double bonds must remain constant in both intact lipids, so as to show the same mass, they were assigned accordingly. Based on the mass there were a total of 34 carbons (18 in one, 16 in the other) and 5 double bonds present in both lipids' fatty acids, therefore one lipid must be $C_{18:3}/C_{16:2}$ while the other must be $C_{18:2}/C_{16:3}$.

DISCUSSION

H. ostrearia and *N. perminuta* stand out as the two diatom species to contain primarily C_{18}/C_{16} and C_{18}/C_{18} forms of MGDG and DGDG. We did not detect any C_{18} fatty acids in the remaining two species, *S. marinoi* or *T. weissflogii*, and only very small

amounts of C_{18} in *P. tricornutum*. This indicates a difference in these diatoms resulting from a possible preference of biosynthetic pathways for these lipids (see below), and could also be linked to a difference in plastid ancestry. According to a study by Pillet et al. (2010), the plastids of the genera *Haslea, Navicula,* and *Phaeodactylum* were shown to be closely related by a phylogeny created using chloroplast 16S rDNA, and a study by Pniewski et al. (2010) showed the three diatom species also to be closely related using 18S rDNA.

Similar to the previous studies by Yan et al. (2011) and Yongmanitchai and Ward (1993) we also found that both *S. marinoi* and *P. tricornutum* contained predominantly C_{20} and C_{16} fatty acids in their galactolipids. While the Yongmanitchai and Ward (1993) study used reverse-phase HPLC, positive-ion ESI/MS/MS, a newer technology not present at that time, should allow a better examination of the intact forms of MGDG and DGDG of this species and provide a more reliable view of its plastid lipid profile, particularly its regiochemistry. According to their results, *P. tricornutum* contained primarily $C_{20:5}/C_{16:4}$, $C_{20:5}/C_{16:1}$, and $C_{16:1}/C_{16:1}$ forms of MGDG and $C_{20:5}/C_{16:1}$ as its primary form of DGDG. Yan et al. (2011) studied three strains of the genus *Skeletonema,* two of which they referred to as *S. costatum* based solely on their morphologies and lipid profiles. Those two strains are shown to have different plastid lipid profiles, with one of them even showing some C_{18}/C_{16} forms of MGDG, which we were unable to find. The strain SKSPXS0711 contained $C_{16:1}/C_{16:3}$, $C_{16:3}/C_{16:3}$, and $C_{20:5}/C_{16:3}$ as its primary forms of MGDG, and $C_{20:5}/C_{16:2}$ as its primary form of DGDG. The other strain, SCXMBO2, contained $C_{16:2}/C_{16:0}$, $C_{16:2}/C_{16:2}$, and $C_{20:5}/C_{16:3}$ as its primary forms of MGDG, and 4 forms of DGDG with very similar total amounts:

$C_{14:0}/C_{16:1}$, $C_{16:1}/C_{16:1}$, $C_{16:0}/C_{16:1}$, and$C_{20:5}/C_{16:2}$. Yan et al. (2011) were unable to classify the species of the third strain, but found that its lipid profile differed significantly enough from the other two strains to hinder its naming as *S. costatum*. This third strain contained near equal amounts of four forms of MGDG – $C_{16:2}/C_{16:0}$, $C_{16:0}/C_{16:1}$, $C_{20:4}/C_{16:2}$, and $C_{20:5}/C_{16:1}$ – and near equal amounts of four forms of DGDG – $C_{16:1}/C_{16:1}$, $C_{20:5}/C_{16:2}$, $C_{20:4}/C_{16:2}$, and $C_{20:4}/C_{16:1}$.

Although it is influenced by other factors, such as growth temperature (Vieler et al., 2008) and inter- or intra-species variability (Yan et al., 2011), the difference in MGDG and DGDG composition seen here between the examined diatom species containing primarily C_{20}/C_{16}, and those containing primarily C_{18}/C_{16} forms of these lipids resembles the split seen by Gray et al. (2009) in peridinin-containing dinoflagellates with C_{20}/C_{18} and another group of peridinin-containing dinoflagellates with C_{18}/C_{18} forms of MGDG and DGDG. The diatom plastid is of red algal origin, as is the plastid of peridinin-containing dinoflagellates, but very little study has been done on the fatty acid regiochemistry of red algae MGDG and DGDG. Generally, red algae do not produce high amounts of unsaturated C_{20} fatty acids, except for the Cyanidales family that contains the model red alga *Porphyridium cruentum* (Khozin et al., 1997; Sato et al., 2007). A study by Adlerstein et al. (1997) was able to show that a free-living red alga, such as *P. cruentum*, and by extension the red algal plastid of both the C_{18}/C_{16} and C_{20}/C_{16} diatoms and C_{20}/C_{18} peridinin-containing dinoflagellates, is able to produce large amounts of both $C_{20:5}/C_{20:5}$ MGDG and $C_{20:5}/C_{20:5}$ DGDG. The appearance of differences in MGDG and DGDG fatty acid composition may be indicative of a difference in red algal plastid ancestry. While we are unable to rule out the possibility that lipid

biosynthesis genes derived from other groups of algae that may have been obtained earlier in the diatoms' evolutionary history have participated in the synthesis of these unsaturated C_{20} fatty acids, we hypothesize that the presence of these $C_{20:5}$ fatty acids seen in *S. marinoi, T. weissflogii, P. tricornutum, H. ostrearia* and *N. perminuta* was obtained from their red algal plastid ancestor.

In higher plants, the synthesis of galactolipids proceeds through two pathways, one prokaryotic and one eukaryotic, mainly differing by the type of fatty acids at the *sn*-1 and *sn*-2 positions and their site of synthesis in the cell (Browse et al., 1986). The prokaryotic pathway occurs entirely in the chloroplast and biosynthesizes galactolipids including MGDG and DGDG in a *de novo* fashion, which contain C_{18} in the *sn*-1 position and C_{16} in the *sn*-2 position. In the eukaryotic pathway $C_{18:1}$ is incorporated into phospholipids, which is localized to the endoplasmic reticulum. Phospholipids release diacylglycerols typically and characteristically comprised of $C_{18:2}$/$C_{18:2}$ and $C_{16:0}$, which are transported to the chloroplast and used to produce MGDG and DGDG and subsequently modified by desaturases. In most higher plants, galactolipids synthesized according to the eukaryotic pathway contain predominantly C_{18} fatty acid in both *sn*-1 and *sn*-2 positions, whereas in some primitive angiosperm families, C_{16} can be found in the *sn*-2 position (Browse et al., 1986).

However, galactolipid synthesis in algae is more complicated than what is observed in the higher plants. In microalgae, very long chain polyunsaturated fatty acids including eicosapentaenoic acid (EPA; $C_{20:5)}$ and arachidonic acid (AA; $C_{20:4}$) are also incorporated into their galactolipids (Khozin-Goldberg et al., 2002; Schneider et al., 1995). Previous radiolabelling studies on the production of EPA in the diatom, *P.*

tricornutum, proposed that four different pathways were involved in the production of EPA (Arao et al., 1994). It has been shown that EPA can be contributed to MGDG synthesis from phospholipids or even triacylglycerides (Khozin-Goldberg et al., 2000; Khozin-Goldberg et al., 2002). In microalgae, galactolipids have also been observed with both prokaryotic and eukaryotic fatty acid species (C_{20}/C_{16}) (Khozin-Goldberg et al., 2002). Radiolabelling studies of the eustigmatophyte, *Monodus subterraneus*, and the diatom, *P. tricornutum* have shown that EPA is synthesized in the ER as part of phospholipids (Domergue et al., 2003). The EPA can then be transported into the plastid and incorporated at the *sn*-1 position to form the galactolipids, while the C_{16} at the *sn*-2 position is synthesized in the plastid. Domergue et al. (2003) has also hypothesized that EPA could either be transported into the plastid as a free fatty acid or as part of a diacylglycerol moiety. In the present study, three of the diatoms (*S. marinoi*, *T. weissflogii*, and, *P. tricornutum*) contained galactolipids primarily comprised of C_{20}/C_{16} fatty acids, which could result from the activation of both the prokaryotic and the eukaryotic pathways. They were also found to produce minor forms of C_{16}/C_{16} which are likely synthesized through the prokaryotic pathway in the plastid. However, the other two diatoms, *N. perminuta* and *H. ostrearia* analyzed in this study had galactolipids primarily comprised of lipids produced in the plastid. The C_{18}/C_{16} fatty acid species are primarily known to be synthesized in the plastid as part of the prokaryotic pathway with the final product being dependent on the desaturases present in the plastid. However, recent studies have also illustrated that C_{18}/C_{16} containing galactolipids can be coordinated through both the eukaryotic and prokaryotic pathways (Sato & Moriyama, 2007). These pathways can be elucidated through future radiolabeling studies in diatoms.

H. ostrearia and *N. perminuta* did make 15% and 13% respectively of $C_{20:5}/C_{16:3}$ MGDG, which as discussed above is a product of both eukaryotic and prokaryotic pathways. The eventual development and analysis of transcriptomic databases for *H. ostrearia*, *N. perminuta*, *S. marinoi*, and *T. weissflogii* will aid in the further elucidation of galactolipid biosynthesis pathways, and will also facilitate comparisons with another *Thalassiosira* species, *T. pseudonana*, and *P. tricornutum*, two species for which already exist abundant transcriptomic data (Armbrust et al., 2004; Bowler et al., 2008).

A phylogeny constructed from 18S rRNA of many species of diatoms shows that these five diatoms fall into two groups, which also seem to be correlated to morphology (Rampen, 2009). *N. perminuta* and *H. ostrearia* are closely related and fall into a clade with *P. tricornutum* that is composed of the pennate diatoms while *Skeletonema* species and *T. weissflogii* are closely related quite removed from the other three and occur at an earlier branching point in a clade composed of the centric diatoms (Rampen, 2009). The relatedness of *N. periminuta* to *H. ostrearia* and *S. marinoi* to *T. weissflogii* can also be seen in their MGDG and DGDG composition. *P. tricornutum* shares MGDG and DGDG composition with the two examined centrics but, as stated above, has been shown to be more related to the pennates. Further radiolabelling studies need to be done to confirm that galactolipid synthesis is similar to other microalgae examined. It will also provide knowledge of which donor molecule (phospholipid or triacylglyceride) provides EPA to the plastid for incorporation into MGDG and DGDG. Genetic studies of these species will better elucidate the pathways by providing evidence of the desaturases present in both prokaryotic and eukaryotic pathways.

Centric and pennate diatoms, which are mostly pelagic and benthic organisms respectively, also differ regarding their photoprotection capacity against sudden increase in irradiance (Lavaud et al., 2007). On the short-term, non-radiative dissipation of excess energy is an important component for photoprotection. This mechanism involves the operation of the xanthophyll cycle, which, in diatoms, basically consists in the conversion in high light of diadinoxanthin (DD) to its de-epoxidized form, diatoxanthin (DT), due to the activation of a diadinoxanthin de-epoxidase (DDE) by the acidification of the thylakoid lumen (Lavaud, 2007). An important factor for DDE activity is the presence of MGDG in the thylakoid membrane (Wilhelm et al., 2006). MGDG favors the solubilization of DD and participates in the formation of three-dimensional structures, providing a preferred docking site for the activated enzyme and facilitating DD de-epoxidation to DT by DDE (Goss et al., 2005). It is worthy to note that DGDG and particularly SQDG, another important constituent of thylakoid membrane in diatoms (Vieler et al., 2007), are also involved in the formation of the three-dimensional structures and in the stabilization of these 'lipid macrodomains' (Goss et al., 2009). Differences in the photoprotection strategies observed between centric and pennate diatoms (Grouneva et al., 2009; Lavaud et al., 2007;) could thus originate from the lipid composition (relative proportion of MGDG, DGDG, SQDG; fatty acid profile; regiochemistry) and from the architecture of their thylakoid membrane (formation of three-dimensional structures; stabilization of lipid macrodomains), a point that deserves more study.

Regarding *H. ostrearia'*s ability to synthesize marennine, it may provide another opportunity for more study. It has been shown that marennine may have biological

activities, such as antibacterial and antiviral properties (Gastineau et al., 2012), and free radical scavenging (Pouvreau et al., 2008). The antioxidant and free radical scavenging properties of this pigment may indicate a role in photoprotection of the diatom as marennine production has been linked to environmental stress, such as high irradiance and low nutrients, and coincides with a rearrangement of thylakoid number and the thylakoid membranes themselves, but it is unknown if this rearrangement is a consequence of the environmental conditions or of the pigment production itself (Nassiri et al., 1998). The plastid lineage of this "blue" diatom is currently unknown. Future studies are planned to examine the modifications made to the thylakoid galactolipids of diatoms under various growth conditions, such as light intensity and temperature, and with a particular interest in how different levels of marennine production may affect these lipids in *H. ostrearia*.

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CHAPTER II

THE LONG AND SHORT OF IT: TEMPERATURE-DEPENDENT MODIFICATIONS OF FATTY ACID CHAIN LENGTH AND UNSATURATION IN THE GALACTOLIPID PROFILES OF THE DIATOMS *HASLEA OSTREARIA* AND *PHAEODACTYLUM TRICORNUTUM*

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INTRODUCTION

Diatoms are one of the largest and most varied groups of primary producers on the planet (Moustafa et al., 2009; Seckbach & Kociolek, 2011). They are responsible for over 40% of marine primary production, and 20 to 25% of total primary production (Nelson et al., 1995; Werner, 1977). In all photosynthetic organisms (cyanobacteria, algae, plants), fatty acid composition is important for stabilizing the membrane proteins associated with photosynthesis, such as the light-harvesting complexes and photosystems (Dörmann & Benning, 2002; Mironov et al., 2012; Mizusawa & Wada, 2012) and membrane fluidity is generally involved in the responses to temperature changes in the environment (Los & Murata, 2004; Mikami & Murata, 2003). Studies relating to the modulation of the membrane fatty acids as a function of temperature have been performed on different photosynthetic microorganisms, such as the chlorophyte *Dunaliella salina* (Lynch & Thompson, 1982; 1984), the dinoflagellate *Gyrodinium aureolum* (Parrish et al., 1993), and the haptophyte *Pavlova lutheri* (Tatsuzawa & Takizawa, 1995). For instance, an increase in polyunsaturated fatty acids (PUFAs), a decrease in monogalactosyldiacylglycerol (MGDG) and almost no changes in digalactosyldiacylglycerol (DGDG) were observed in *P. lutheri* grown at 15°C versus 25°C (Tatsuzawa & Takizawa, 1995). Specific studies dealing with the effect of temperature changes on the membrane fatty acid profile of diatoms in particular are lacking, and understanding the lipid biochemistry, with an emphasis on fatty acid regiochemistry, of these photosynthetically important organisms may provide insights into specific lipid-based survival strategies during changing environmental conditions.

Thompson et al. (1992) examined the fatty acid composition of eight marine phytoplankton, including the diatoms *Chaetoceros calcitrans, C. gracilis, C. simplex, Phaeodactylum tricornutum,* and *Thalassiosira pseudonana,* and saw a significant inverse relationship between the concentration of PUFAs and temperature, as did Renaud et al. (1995), in the diatoms *Nitzschia closterium* and *N. paleacea*. This could be considered a general trend, since in phylogenetically distinct groups of algae, total fatty acids and cellular PUFA content have been shown to be influenced by temperature changes. In the eustigmatophyte *Nannochloropsis salina,* Hoffmann et al. (2010) showed that total PUFA proportion, and in particular the long chain eicosapentaenoic acid (EPA, $C_{20:5}$) content, which has an important value for aquaculture and human health, increased at low temperature (17°C, compared with 21 and 26°C), although, when compared with other results obtained using this species or others within the same genus (Olofsson et al., 2012 and references therein), these authors emphasized that the influence of temperature seemed to be complex and difficult to explain.

The major problem with these studies on total fatty acids is that it is unknown what fatty acids are attached to which lipid headgroups, let alone the regiochemistry of the intact lipids. Rousch et al. (2003) used gas chromatography/mass spectrometry (GC/MS) to examine the changes in fatty acid methyl esters (FAMEs) due to changes in growth temperatures in the diatoms *P. tricornutum* and *C. muelleri*, and found that certain fatty acids, such as EPA, $C_{16:1}$, and $C_{18:1}$, decreased as temperature increased, while others, such as $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, and $C_{18:2}$, increased with temperature. Leblond et al. (2010) examined the galactolipid composition of six isolates from the dinoflagellate genus *Pyrocystis* grown under three temperatures (15°C, 20°C, and 25°C) using positive-ion electrospray

ionization/mass spectrometry/mass spectrometry (ESI/MS/MS), which determined the intact lipid as well as the regiochemistry of the lipids. Leblond et al. (2010) found that DGDG tended to be more responsive to changes in temperatures (more unsaturated forms of the *sn*-2 fatty acid were found at lower temperatures) than did MGDG. There is, however, variation in other photosynthetic organisms, such as the red alga *Porphyridium cruentum*, as to which fatty acid (*sn*-1 and/or *sn*-2) and which lipid (MGDG and/or DGDG) was modified in response to temperature (Adlerstein et al., 1997).

Using ESI/MS/MS to study diatom galactolipid composition, it was previously found that the "blue" diatom *Haslea ostrearia* contained primarily C_{18}/C_{16} forms of both MGDG and DGDG, with minor amounts of C_{20}/C_{16}, and was similar to another pennate diatom, *Navicula perminuta* (Dodson et al., 2013). The pennate diatom *P. tricornutum*, conversely, contained primarily C_{20}/C_{16} forms of MGDG and DGDG, with minor amounts of C_{16}/C_{16} and C_{18}/C_{16}, which was similar to the centric diatoms *Skeletonema marinoi* and *Thalassiosira weissflogii* (Dodson et al., 2013).

Haslea ostrearia is well known in France for the production of the water-soluble, non-photosynthetic blue pigment marennine, which causes the greening of oyster ponds and oysters (Gastineau et al., 2012; Nassiri et al., 1998). *Phaeodactylum tricornutum* has become a model organism for diatom research since it is one of only two diatoms to have its genome sequenced and published, the other being *T. pseudonana* (Maheswari et al., 2005). *Phaeodactylum tricornutum* has since been used to study carbohydrate metabolism (Kroth et al., 2008), growth rate (Pérez et al., 2008), and lipid accumulation (Valenzuela et al., 2012). Since *H. ostrearia* and *P. tricornutum* differ in their galactolipid composition at 20°C (Dodson et al., 2013), this work was done in order to determine the method of

temperature acclimation to 30°C as it relates to these two pennate diatoms' galactolipids. *Phaeodactylum tricornutum* is usually considered a pelagic organism, whereas *Haslea ostrearia* is a tychopelagic diatom (Robert 1983), an organism that can be benthic or epiphytic, but also planktonic (Round et al., 1990). *Haslea ostrearia* is euryhaline (Neuville & Daste, 1978; Wraige et al., 1998), and can develop in high light environments (Mouget et al., 1999), thus it seems well adapted to oyster ponds, characterized by shallow and nutrient-rich water, where it mainly proliferates in spring and autumn (Maestrini & Robert, 1981; Robert, 1983). According to the literature, *H. ostrearia* lives mainly in temperate to tropical waters (for a review, see Gastineau et al., 2013), thus acclimation to 30°C could represent a stress relevant enough to study algal exacerbated responses to a water temperature rise.

Unlike previous studies on diatom lipids, this study uses ESI/MS/MS to examine just what modifications are made to specific lipids, and to what positions (*sn*-1 or *sn*-2) those modifications are made. Indeed, what seems to be the case is that both species of diatoms displayed a similar response to temperature. Both *H. ostrearia* and *P. tricornutum* contained C_{20} fatty acids in their forms of MGDG and DGDG when grown at 20°C; however, when grown at 30°C these C_{20} fatty acids were replaced by C_{18} fatty acids at the *sn*-1 position.

MATERIALS AND METHODS

Cultures and growth conditions

Phaeodactylum tricornutum Bohlin CCMP 1327 was obtained from the National Center for Marine Algae and Microbiota (NCMA, East Boothbay, Maine, USA). *Haslea*

ostrearia (Gaillon) Simonsen NCC 344 was obtained from the Nantes Culture Collection (NCC, Université de Nantes, 2 Rue de La Houssiniere, 44322 Nantes, France). Each strain was grown in triplicate in 2L of L1 medium in separate incubators at 20°C and 30°C, an irradiance of ~50 μmol photons · m^{-2} · s^{-1}, and a light:dark cycle of 14:10 hours; they were harvested according to the methodology of Leblond & Chapman (2000).

Lipid extraction and fractionation

Total lipids were extracted, and galactolipids separated from other lipid classes according to the techniques described by Leblond and Chapman (2000). Briefly, the total lipid extracts were separated into five component lipid fractions on columns of activated Unisil silica (1.0 g, 100–200 mesh, activated at 120C; Clarkson Chromatography, South Williamsport, PA, USA). The following solvent regime was used to separate lipids according to polarity, with fraction 5 eluting the most polar lipids (Leblond and Chapman 2000): (1) 12 mL methylene chloride (sterol esters), (2) 15 mL 5% acetone in methylene chloride with 0.05% acetic acid (free sterols, tri- and diacylglycerols, and free fatty acids), (3) 10 mL 20% acetone in methylene chloride (monoacylglycerols), (4) 45 mL acetone (chloroplast glycolipids, including MGDG, DGDG, and sulfoquinovosyldiacylglycerol [SQDG]), and (5) 15 mL methanol with 0.1% acetic acid (polar lipids, including phosphorous- and non-phosphorous-containing lipids).

Mass spectrometry of lipids

Following the procedure of Gray et al. (2009), the lipids of fraction 4 were suspended in methanol, chloroform, and 50 mM sodium acetate (Welti et al., 2002) prior to examination via mass spectrometry. The resulting MGDG and DGDG sodium adducts were analyzed using positive-ion ESI/MS full scans from 100 to 2,000 Da through direct

injection (5 μL sample volume into a methylene chloride carrier solvent at 0.5 $mL \cdot min^{-1}$) into a Finnigan DecaXP ion trap mass spectrometer (Waltham, MA, USA). The abundance of each lipid was determined by calculating relative percent composition based on peak height of raw data in the positive-ion ESI/MS full-scan mode. Subsequent positive-ion ESI/MS/MS was performed using collision energy between 37.5% and 48.0%, and major cleaved fatty acids were identified by the differences between the masses of the original ions and their fragments. Regiochemistry was determined by positive ESI/MS/MS according to the methodology of Guella et al. (2003). Statistical significance between treatments was determined using ANOVA and Tukey's test ($p<0.05$) in SigmaPlot version 12.3 (Systat Software, San Jose, CA).

RESULTS

The most obvious response to temperature between the galactolipid profiles of both *H. ostrearia* and *P. tricornutum* was the complete lack of any C_{20} fatty acids, EPA or arachidonic acid (AA, $C_{20:4}$), in the cultures grown at 30°C. TheMGDG and DGDG forms in *P. tricornutum* that showed significant differences between growth temperatures can be seen in Figure 1. Significantly different MGDG forms of *H. ostrearia* can be seen in Figure 2. *Phaeodactylum tricornutum*'s most abundant form of MGDG at 20°C was a $C_{20:5}/C_{16:3}$ (*sn*-1/*sn*-2, *m/z* 793) at an average of 23% relative abundance, however forms of MGDG with C_{20} fatty acids at *sn*-1 seen at 20°C were completely absent at 30°C. Instead, the most abundant form of MGDG at 30°C became a $C_{18:2}/C_{16:2}$ form of MGDG at *m/z* 773 at 18.2% relative abundance. There were also a number of other C_{18}/C_{16} forms of MGDG that were present in the 30°C cultures, but not the 20°C cultures. There was also a

noticeable difference in the DGDG forms present in *P. tricornutum*. The 2 most abundant forms of DGDG in the 20°C cultures were $C_{16:1}/C_{16:0}$ (*m/z* 913) and $C_{16:1}/C_{16:1}$ (*m/z* 911) forms at 6.4% and 5.8% relative abundance respectively. The two most abundant forms of DGDG in the 30°C cultures, however, were an $C_{18:2}/C_{16:0}$ form (*m/z* 939; seen in Figure 3b) at 6.52% relative abundance and an $C_{18:2}/C_{16:2}$ form (*m/z* 935) at 3.9% relative abundance. There were also 4 forms of DGDG with C_{20} fatty acids at *sn*-1 in *P. tricornutum* at 20°C that were not seen in the cultures grown at 30°C.

Just as was seen in *P. tricornutum*, forms of MGDG and DGDG that contained C_{20} fatty acids seen in the 20°C *H. ostrearia* cultures were also absent from the 30°C cultures. The most abundant form of MGDG in *H. ostrearia* when grown at 20°C was a $C_{18:3}/C_{16:3}$ form (*m/z* 769; seen in Figure 3a) at 39.1% relative abundance. When grown at 30°C, however, the most abundant MGDG form became $C_{18:2}/C_{16:2}$ (*m/z* 773) at 23.8% relative abundance. There were also a number of MGDG forms containing C_{18}/C_{16} fatty acids that were present when grown at 30°C, but not present when grown at 20°C. Regarding the DGDG profile, there was a decrease in the amount of $C_{18:2}/C_{16:0}$ (*m/z* 939), $C_{18:3}/C_{18:1}$ (*m/z* 963), and $C_{18:2}/C_{18:2}$ (*m/z* 963) in cells grown at 20°C compared to 30°C.

Figures 4 and 5 show total carbons and unsaturations for all fatty acids of MGDG and DGDG forms detected at both growth temperatures for *P. tricornutum* and *H. ostrearia* respectively. It is apparent

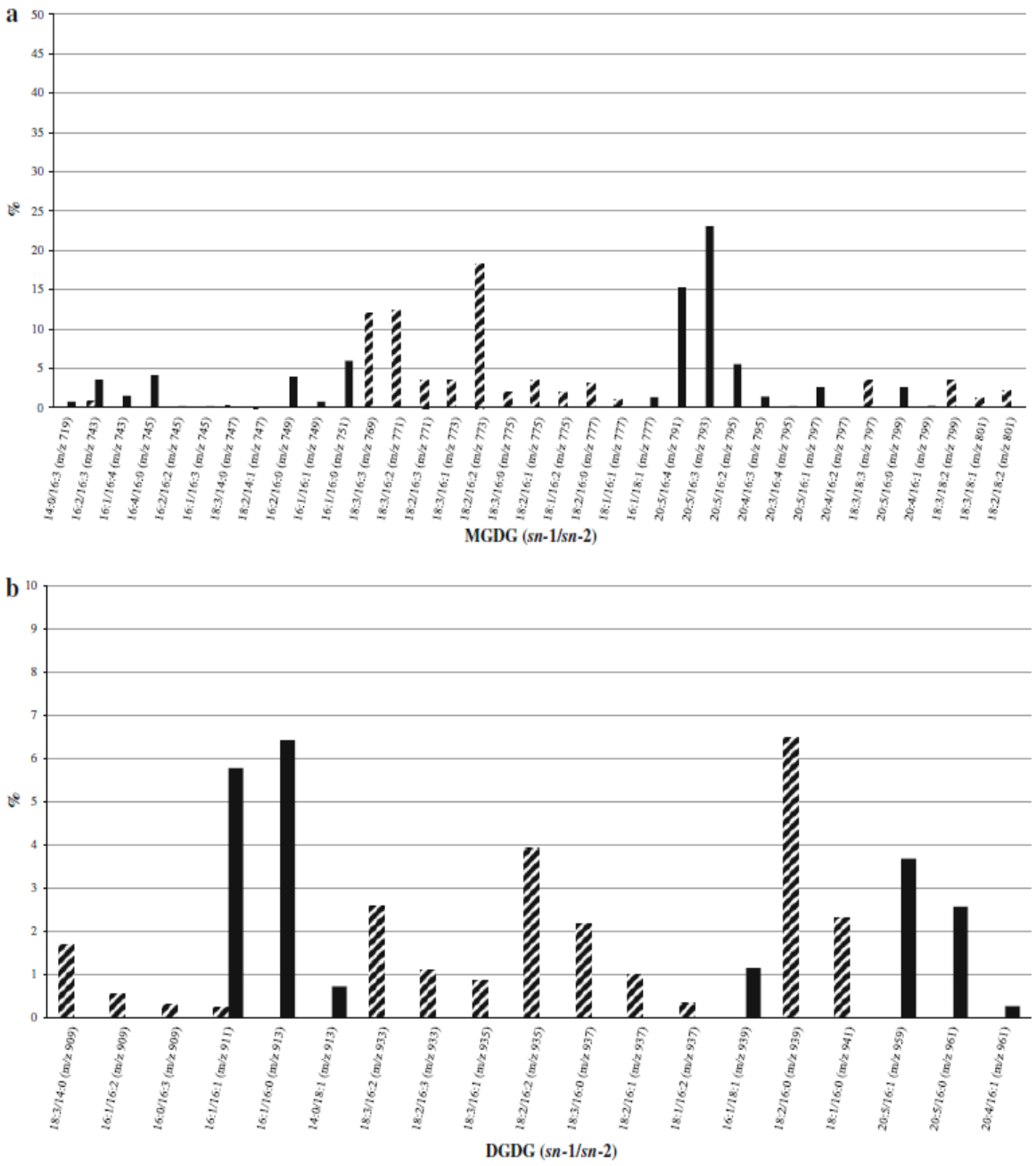

Fig. 1 Relative abundances of significantly different galactolipids with regiochemistry (*sn*-1/*sn*-2) and *m/z* ratio of **a** MGDG and **b** DGDG forms of *P. tricornutum* when grown at 20°C (*solid bars*) and 30°C (*hashed bars*) (standard deviations can be found in Online Resource 1)

in both figures that both species tended to possess longer carbon chains and more unsaturations in their MGDG and DGDG forms when grown at 20°C than when grown at 30°C. It is also evident that at 20°C, both species of diatoms contained galactolipids with the shorter chain fatty acids. In fact, at the higher growth temperature, both diatom species were rich in both MGDG and DGDG forms with 34 carbons in both fatty acids. At the lower growth temperature, however, *P. tricornutum* was rich in MGDG and DGDG with 36 carbons, while *H. ostrearia* only possessed MGDG with 36 carbons. No galactolipid in either species possessed more than 6 combined unsaturations in both fatty acids when grown at 30°C, yet reached up to 8 or 9 unsaturations when grown at lower temperatures.

Significant differences between experimental treatments were seen in a majority of both species' galactolipid forms. Only 3 forms of MGDG and 3 forms of DGDG were not statistically significant in the *P. tricornutum* samples – $C_{14:0}/C_{16:1}$ MGDG, $C_{16:1}/C_{16:2}$ MGDG, $C_{16:0}/C_{16:3}$ MGDG, $C_{18:2}/C_{14:0}$ DGDG, $C_{16:0}/C_{16:2}$ DGDG, and $C_{20:5}/C_{16:2}$ DGDG – and of these 6 galactolipids, only $C_{16:1}/C_{16:2}$ MGDG and $C_{16:0}/C_{16:3}$ MGDG were present at both growth temperatures. The galactolipids without significant differences that were only present at 30°C were $C_{14:0}/C_{16:1}$ DGDG and $C_{20:5}/C_{16:2}$ DGDG, while $C_{18:2}/C_{14:0}$ DGDG and $C_{16:0}/C_{16:2}$ DGDG were only present at 20°C. There were 19 galactolipid forms found in *H. ostrearia* that were not significantly different between the two growth temperatures, 6 MGDG forms and 13 DGDG forms, and of these molecular forms 10 were present at both growth temperatures. Only 3 forms of DGDG were significantly different in *H. ostrearia* between the two growth temperatures – $C_{18:2}/C_{16:3}$, $C_{18:3}/C_{18:1}$, and $C_{18:2}/C_{18:2}$ – with all of them present at less than 2% at 30°C and not present at all at 20°C.

The statistical analyses of each lipid, including standard deviations as well as individual relative percent abundances for each replicate of *P. tricornutum* and *H. ostrearia* has been included as Appendices 1 and 2, respectively.

Overall, when grown at 30°C, the MGDG to DGDG ratio increased from 3.1 ± 1.3 to 3.4 ± 0.8 in *P. tricornutum*, and from 3.7 ± 0.8 to 9.4 ± 6.2 in *H. ostrearia* from what was observed at 20°C. In both species, the ratios reflect a decrease in MGDG coupled to an increase in DGDG, when grown at the higher temperaure. MGDG decreased from 76.7 to 74.0 and DGDG increased from 23.3 to 26.0 % of total galactolipids in *P. tricornutum*. In *H. ostrearia*, the corresponding values are 87.9 to 78.1(MGDG) and 12.1to 21.9 (DGDG), respectively.

DISCUSSION

In the present work, positive-ion electrospray ionization/mass spectrometry/mass spectrometry technique was used to specifically study intact lipids and to identify the regiochemistry of the fatty acids attached to of the two types of galactolipids, MGDG and DGDG, in two different pennate diatoms, the model organism *P. tricornutum* and the marennine-producing *H. ostrearia*. Both *H. ostrearia* and *P. tricornutum* demonstrated a complete shift away from C_{20} fatty acids in both MGDG and DGDG when grown at 30°C, eliminating the longest and most unsaturated lipids from their galactolipid profiles. This supports the hypothesis that more saturated lipids will be more prevalent at higher temperatures due to the need for increased membrane melting temperature. Conversely, this shift also eliminates the longest fatty acid chains from the galactolipids, which would be presumed to have the highest melting temperature. It is evident that the loss of C_{20}

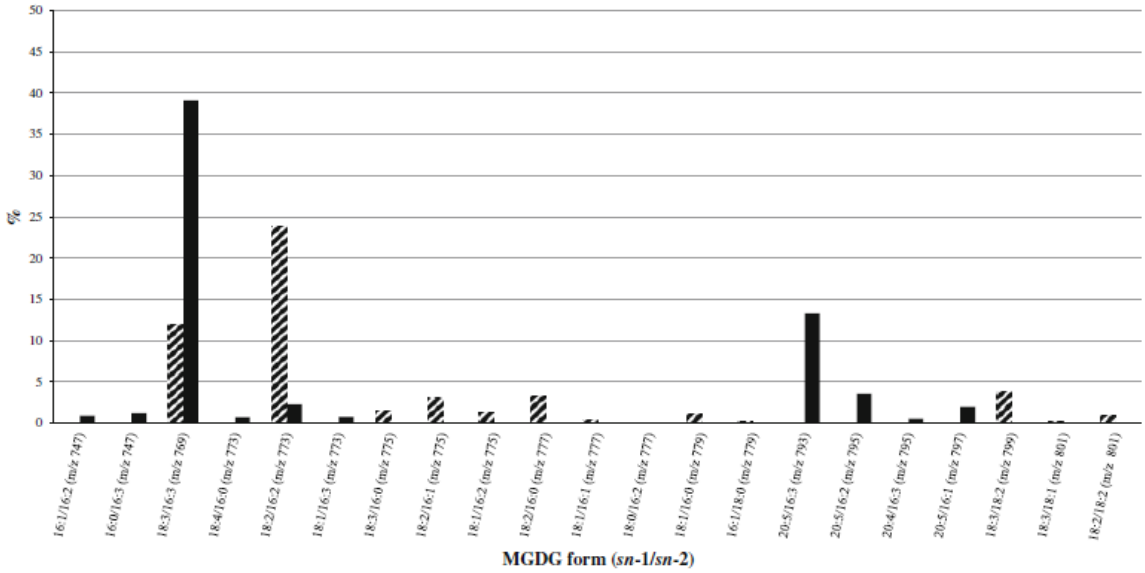

Fig. 2 Relative abundances of significantly different MGDG forms with regiochemistry and *m/z* ratios of *H. ostrearia* when grown at 20°C (*solid bars*) and 30°C (*hashed bars*) (standard deviations can be found in Online Resource 2)

fatty acids from the chains is offset by the correlated loss of many of the shorter chain fatty acids, such as many C_{14} and C_{16} fatty acids which are present at the *sn*-1 position of multiple MGDG forms and a single DGDG form only in the samples grown at 20°C. *H. ostrearia* and *P. tricornutum* did not produce fatty acids with four or more unsaturations when grown at 30°C.

When grown at lower temperatures *H. ostrearia* and *P. tricornutum* exhibited longer fatty acid chains and more unsaturations. This observation is consistent with the interpretation that in order to maintain membrane fluidity at lower temperatures, unsaturations are needed to force spacing between the membrane lipids and decrease the melting temperature of the membrane. Fewer unsaturations and shorter fatty acid chains, seen at the higher temperatures, demonstrate the need for a more tightly packed membrane, reducing the fluidity of the membrane. These changes allow for the membrane to maintain the fluidity or order it requires for a particular growth temperature by maintaining increasing or decreasing its melting temperature, a response observed in almost all photosynthetic organisms (e.g., Mikami & Murata, 2003; Mizusawa & Wada, 2012).

While the total amount of PUFAs, the fatty acid chain length, the level of unsaturation, and the ratio of lipid molecular forms have been observed as general possible lipid modifications in response to environmental change in microalgae, what seems to be especially prevalent with diatom species in response to changes in growth temperature is the alteration of fatty acid chain length and saturation (Chen, 2012; Jiang & Gao, 2004; Renaud et al., 1995; Thompson et al., 1992; this study), but see Boelen et al.

(2013) for a mitigated view. Renaud et al. (2002) examined the lipid content of four tropical algae, the diatom *Chaetoceros* sp., the cryptomonads *Rhodomonas* sp. and *Crytpomonas*, and an unidentified prymnesiophyte, under increasing growth temperatures using GC/MS analysis of FAMEs. All four species examined showed an increase in saturated fatty acids and a decrease in the amount of EPA with increasing temperatures. The present study confirms that levels of EPA decreased in *P. tricornutum* grown under higher temperatures, a result in accordance with Jiang & Gao (2004), and demonstrates that this response is seen in *H. ostrearia* as well.

Contrary to what was seen by Rousch et al. (2003), increasing levels of $C_{14:0}$, $C_{16:0}$, and $C_{18:0}$ fatty acids were not seen in *P. tricornutum* under the higher growth temperature, nor were decreasing levels of $C_{16:1}$ and $C_{18:1}$. These data also show a much greater variety of fatty acids detected compared to the nine that were found by Rousch et al. Rousch et al. (2003), however, examined total FAMEs using GC/MS. It is possible that the changes seen in that study may occur in other lipids outside of the galactolipid fraction examined here. However, the experimental conditions were different between the two studies, for instance regarding the light regime: 30 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$, and a light:dark cycle of 12:12 hours, as compared to 50 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$, and a light:dark cycle of 14:10 hours in the present study. Moreover, Rousch et al. (2003) focused on the changes in FA profiles on the short (24h) and the very short term (2h), and exposed their *P. tricornutum* cultures to temperature stress (up to 30°C and 40°C, respectively), whereas the cultures used in the present study were acclimated to the two growth temperatures (20°C and 30°C). These differences in experimental set up, especially the time lapse for temperature

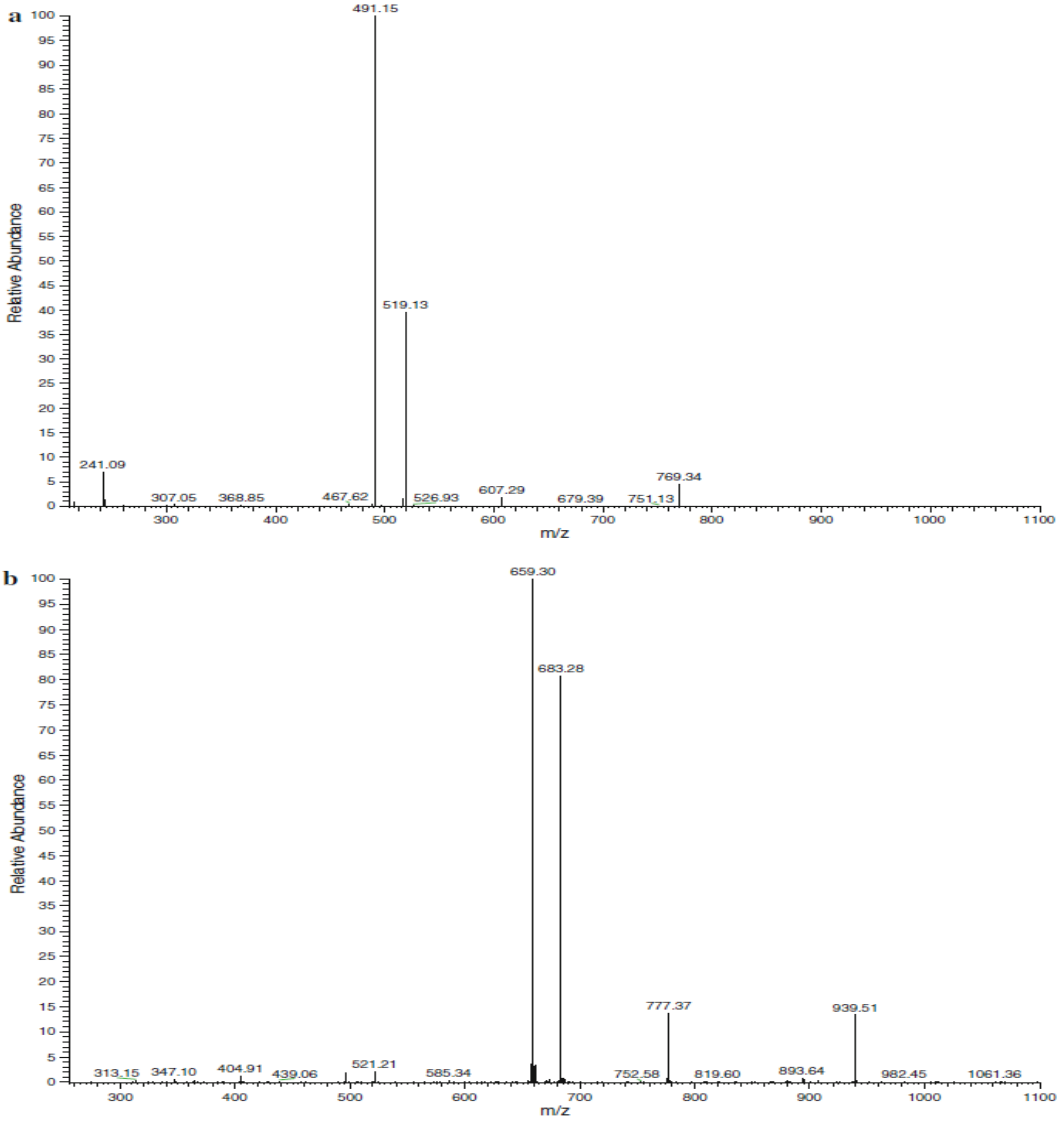

Fig. 3 Positive-ion electrospray ionization/mass spectrometry/mass spectrometry (ESI/MS/MS) spectra of two galactolipids: **a** $C_{18:3}/C_{16:3}$ monogalactosyldiacylglycerol (MGDG) (molecular ion of *m/z* 769) from *H. ostrearia* grown 20°C, also seen at 30°C. The *m/z* 491 fragment represents the lipid minus the $C_{18:3}$ fatty acid from the *sn*-1 position, and the *m/z* 519 fragment represents the lipid minus the $C_{16:3}$ fatty acid from the *sn*-2 position; **b** $C_{18:2}/C_{16:0}$ digalactosyldiacylglycerol (DGDG) (*m/z* 939) from *P. tricornutum* grown at 30°C. The *m/z* 659 fragment represents the lipid minus the $C_{18:2}$ fatty acid from the *sn*-1 position, and the *m/z* 683 fragment represents the lipid minus the $C_{16:0}$ fatty acid from *sn*-2 position. The minor *m/z* 777 fragment represents the loss of one of the galactose molecules from the *sn*-3 head group

acclimation (see Lynch & Thompson, 1984) do not allow further comparison of the two studies. More generally, two recent studies illustrate the difficulties encountered when comparing data obtained using different experimental devices, parameters, and species. Chen (2012) studied the total lipids and FA composition of 12 diatom species grown under various combinations of temperatures and light intensities, corresponding to ambient conditions in summer and winter (for instance, temperature ranging from 23.5 to 38°C, and 11.5 to 25°C, respectively), and in a constant incubator (24°C, 12:12h light:dark cycle, 122 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$). Chen (2012) showed that the FA composition could be affected by temperature only, light only, or by both temperature and light, and that responses were species-dependent. On the other hand, Boelen et al. (2013) compared EPA production in a polar (*Chaetoceros brevis*) and a temperate diatom (*T. weissflogii*) grown at control (3°C and 16°C, respectively), and 'high' temperature (7°C and 20°C, respectively). Possibly due to the small range of temperature changes tested for each species, in contrast to Chen (2012), these authors observed no significant effect of temperature on EPA content (expressed on an algal biovolume basis), whatever the irradiance (10, 25, 75 and 150 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$) .

Similar, contrasting, or variable effects of temperature on lipid profiles have been observed in phylogenetically different algae. For instance, in the eustigmatophyte *Nannochloropsis* sp., the total EPA content decreased with increasing temperature (Hoffman et al., 2010). Moreover, the ratio of FAs in the *sn-1* and *sn-2* positions of MGDG changed with temperature, mainly due to the percentage of the form $C_{20:5}/C_{20:5}$ MGDG (Sukenik et al., 1993) which significantly decreased with increasing temperature, as it did in the red alga *P. cruentum* (Adlerstein et al., 1997). Flaim et al. (2012) also

observed a shift in the lipid forms produced by the cold-adapted dinoflagellate *Borghiella dodgei*. While *B. dodgei* possessed primarily $C_{18:5}$ and $C_{18:4}$ fatty acids in its MGDG and DGDG forms, these lipids decreased as growth temperature increased and were replaced by other less unsaturated fatty acids. The authors also posit that by reducing the activity of the desaturases required to make those polyunsaturated fatty acids, *B. dodgei* was conserving energy at higher growth temperatures (Flaim et al., 2012). In other dinoflagellates, Leblond et al. (2010) examined temperature dependent modifications of the galactolipid profile of members of the genus *Pyrocystis*, and observed that only the *sn*-2 fatty acid of DGDG was modified, while MGDG and trigalactosyldiacylglycerol (TGDG) remained mostly constant. In fact, only one form of MGDG, $C_{20:5}/C_{18:5}$, in *P. lunula* UTEX 2166 showed any response to temperature. Likewise, another study performed on the chromerid, *Chromera velia,* observed that the *sn*-2 fatty acid became less saturated when grown at higher temperatures (Dahmen et al. 2013). Across most galactolipids present in the two species *H. ostrearia* and *P. tricornutum*, the largest modifications appear to occur at the *sn*-1 position of either MGDG or DGDG.

In a previous study, it was shown that *H. ostrearia* and *P. tricornutum* differed regarding their galactolipid profiles (Dodson et al., 2013). The former contained primarily C_{18}/C_{16} forms of both MGDG and DGDG, and the latter primarily C_{20}/C_{16} forms of MGDG and DGDG. The present work shows that these two pennate diatoms respond similarly to changes in growth temperature, with an increase in FA unsaturation and chain length at 20°C in comparison with 30°C, but also an increase in the ratios MGDG to DGDG. However, they differ significantly as to the amplitude of the ratio MGDG to

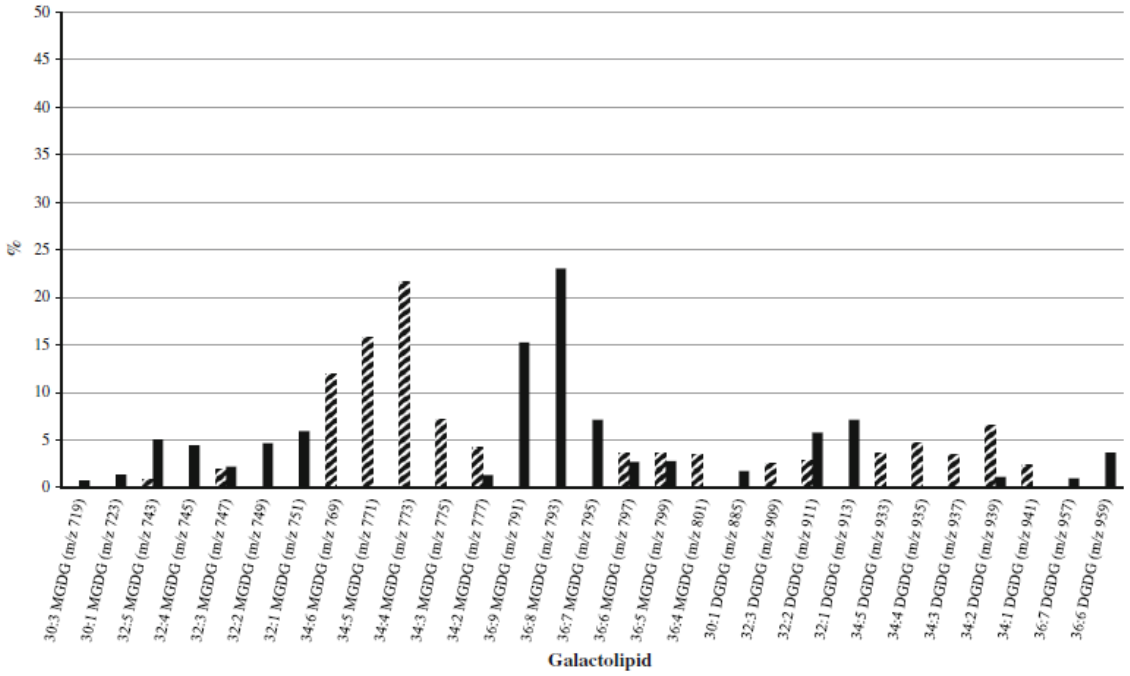

Fig. 4 Total number of carbons and unsaturations for all galactolipids of *P. tricornutum* when grown at 20°C (*solid bars*) and 30°C (*hashed bars*)

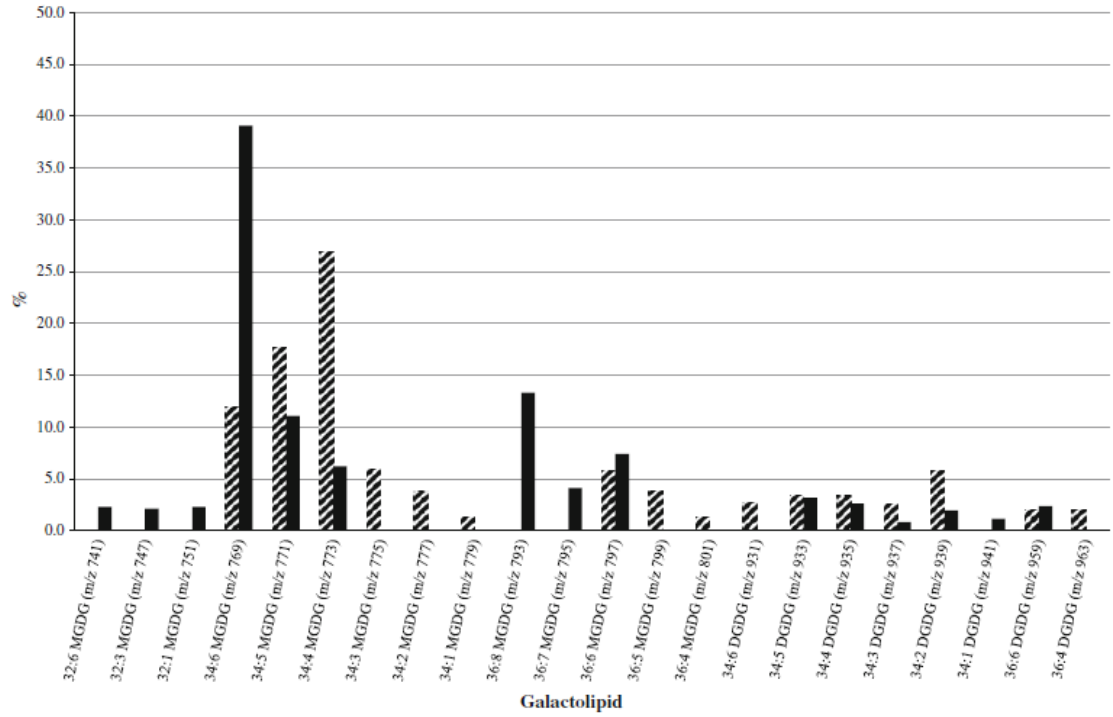

Fig. 5 Total number of carbons and unsaturations for all galactolipids of *H. ostrearia* when grown at 20°C (*solid bars*), and 30°C (*hashed bars*)

DGDG, mostly due to the decrease in DGDG at low temperature, which is > 50% in *H. ostrearia*, as compared to *ca.* 10% in *P. tricornutum*. These changes in galactolipid profiles could reflect differences in their respective strategies of acclimation of the photosynthetic apparatus to the growth temperature, and in their autecology – *P. tricornutum* is usually considered a pelagic organism, while *H. ostrearia* is considered a benthic or epiphytic diatom, although occasionally it is tychopelagic. In the laboratory, *P. tricornutum* can outcompete *H. ostrearia* (J.-L. Mouget, unpublished results), and the former displays two times more nonphotochemical chlorophyll *a* fluorescence quenching (NPQ) than the latter (Rech et al., 2005). This reveals a higher capacity to dissipate overexcitation at the PSII antenna that could result from differences in their galactolipid profiles. Moreover galactolipids in thylakoid membranes, especially MGDG, and to a lesser extent DGDG, play important roles in the response to change in growth temperature in photosynthetic organisms. Their role is structural (increasing the unsaturation of fatty acids allows survival at low temperature, by decreasing the membrane's melting temperature), but also functional (for instance, regarding the operation of the xanthophyll cycle), as far as the two aspects can be dissociated (Goss et al. 2009). The xanthophyll cycle is located in the chloroplast and represents an important component of the photoprotection mechanisms displayed by photosynthetic organisms, by preventing an overexcitation of the PSII reaction centers (Lavaud, 2007). In diatoms, it consists of the conversion in high light of diadinoxanthin (DD) to its de-epoxidized form, diatoxanthin (DT), due to the activation of a diadinoxanthin de-epoxidase (DDE) by the acidification of the thylakoid lumen (Lavaud, 2007). The de-epoxidation of DD to DT results in an increased ability to dissipate excess excitation energy in the antenna complex of PSII.

This photoprotective capacity is closely related to the NPQ, one of the most important mechanism for rapid (seconds to minutes) regulation of photochemistry (Lavaud, 2007).

If in diatoms MGDG and sulfoquinovosyldiacylglycerol (SQDG) are quantitatively the two most important lipids in the thylakoid membranes (Goss et al. 2009), the light-harvesting complexes (LHCs), the fucoxanthin chlorophyll protein complexes (FCPs), are especially enriched in MGDG (Goss et al., 2009). It has been shown in diatoms that MGDG facilitates DD de-epoxidation to DT by DDE (Goss et al., 2005). Furthermore, *in vitro* experimental evidence showed that MGDG molecules do not form lamellar bilayer membranes but specific three-dimensional domains in the thylakoids, which allow hypothesizing that MGDG influences the curvature of the membranes (Goss et al., 2005), and facilitates xanthophyll solubilization and accessibility to the de-epoxidase active sites in the lumen (Schaller et al., 2010), especially at low temperature (Vieler et al., 2008). Moreover, at low temperature, high rates of de-epoxidation were observed in the MGDG-forming domains of the thylakoid membranes (Latowski et al., 2003). Regarding other galactolipid molecules, DGDG is specifically bound to PSII and plays an important role for the stabilization of the oxygen-evolving complex, through the binding of extrinsic proteins on the donor side of PSII (Sakurai et al., 2007). DGDG could also participate in the organization of the thylakoid membrane domains and influence the functioning of PSI and the intersystem electron transport rate (Ivanov et al., 2006).

In this study, the data demonstrate that both *H. ostrearia* and *P. tricornutum* possess similar methods for acclimating their galactolipid profiles to a higher growth temperature. The elimination of the longest, C_{20}, and shortest, C_{14} and C_{16}, fatty acids

from the galactolipids at the higher growth temperature suggests a narrowing of the melting temperature range of these photosynthetically important membranes, and helps preserve the membrane's fluidity at various growth temperatures. The reduction in the number of unsaturations at the higher growth temperature induces a tighter packing of the galactolipids, which also increases the melting temperature of these membranes. Interesting questions raised by this study include whether or not this lipid modification strategy extends to other classes of membrane lipids, such as the phospholipids, and if centric diatoms or other pennate diatoms use these strategies as well.

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APPENDICES

APPENDIX I

Statistics of *P. tricornutum* galactolipids at 20° and 30°C

Relative percent abundances for each replicate in each treatment of *P. tricornutum* , along with averages, standard deviations, and p-values (significant differences in bold).

Galactolipid Species	Replicate 1 20° C	Replicate 2 20° C	Replicate 3 20° C	Mean 20° C	Standard Deviation 20° C	Replicate 1 30° C	Replicate 2 30° C	Replicate 3 30° C	Mean 30° C	Standard Deviation 30° C	P-value
14:0/16:3 (*m/z* 719) MGDG	0.000	0.000	0.000	0.000	0.000	0.625	0.373	1.290	0.762	0.387	**0.049**
14:0/16:1 (*m/z* 723) MGDG	0.000	0.000	0.000	0.000	0.000	2.288	1.572	0.259	1.373	0.840	0.082
16:2/16:3 (*m/z* 743) MGDG	0.695	1.724	0.240	0.886	0.621	2.500	3.748	4.448	3.566	0.338	**0.02**
16:1/16:4 (*m/z* 743) MGDG	0.000	0.000	0.000	0.000	0.000	1.048	1.571	1.864	1.494	0.806	**0.003**
16:4/16:0 (*m/z* 745) MGDG	0.000	0.000	0.000	0.000	0.000	3.272	5.538	3.429	4.080	0.044	**0.005**
16:2/16:2 (*m/z* 745) MGDG	0.000	0.000	0.000	0.000	0.000	0.156	0.263	0.163	0.194	0.049	**0.005**
16:1/16:3 (*m/z* 745) MGDG	0.000	0.000	0.000	0.000	0.000	0.141	0.238	0.147	0.175	1.033	**0.005**
18:3/14:0 (*m/z* 747) MGDG	0.178	0.181	0.352	0.237	0.081	0.000	0.000	0.000	0.000	0.000	**0.015**
18:2/14:1 (*m/z* 747) MGDG	0.054	0.055	0.106	0.072	0.025	0.000	0.000	0.000	0.000	0.000	**0.015**
16:1/16:2 (*m/z* 747) MGDG	0.849	0.859	1.676	1.128	0.388	2.312	1.798	0.803	1.637	0.627	0.384
16:0/16:3 (*m/z* 747) MGDG	0.330	0.334	0.651	0.438	0.151	0.782	0.608	0.271	0.554	0.212	0.564
16:2/16:0 (*m/z* 749) MGDG	0.000	0.000	0.000	0.000	0.000	3.637	3.855	4.221	3.904	0.047	**<0.001**
16:1/16:1 (*m/z* 749) MGDG	0.000	0.000	0.000	0.000	0.000	0.711	0.753	0.825	0.763	0.241	**<0.001**
16:1/16:0 (*m/z* 751) MGDG	0.000	0.000	0.000	0.000	0.000	5.233	5.436	7.188	5.952	0.878	**<0.001**
18:3/16:3 (*m/z* 769) MGDG	12.383	14.671	8.882	11.979	2.381	0.000	0.000	0.000	0.000	0.000	**0.002**
18:3/16:2 (*m/z* 771) MGDG	15.323	15.466	6.238	12.342	4.316	0.000	0.000	0.000	0.000	0.000	**0.016**
18:2/16:3 (*m/z* 771) MGDG	4.280	4.320	1.743	3.448	1.206	0.000	0.000	0.000	0.000	0.000	**0.016**
18:3/16:1 (*m/z* 773) MGDG	2.926	3.119	4.127	3.391	0.527	0.000	0.000	0.000	0.000	0.000	**<0.001**
18:2/16:2 (*m/z* 773) MGDG	15.684	16.720	22.124	18.176	2.824	0.000	0.000	0.000	0.000	0.000	**<0.001**
18:3/16:0 (*m/z* 775) MGDG	1.687	1.730	2.357	1.925	0.306	0.000	0.000	0.000	0.000	0.000	**<0.001**
18:2/16:1 (*m/z* 775) MGDG	2.993	3.069	4.183	3.415	0.544	0.000	0.000	0.000	0.000	0.000	**<0.001**
18:1/16:2 (*m/z* 775) MGDG	1.660	1.701	2.319	1.893	0.301	0.000	0.000	0.000	0.000	0.000	**<0.001**
18:2/16:0 (*m/z* 777) MGDG	2.279	4.876	2.177	3.111	1.249	0.000	0.000	0.000	0.000	0.000	**0.024**
18:1/16:1 (*m/z* 777) MGDG	0.776	1.660	0.741	1.059	0.425	0.000	0.000	0.000	0.000	0.000	**0.024**
16:1/18:1 (*m/z* 777) MGDG	0.000	0.000	0.000	0.000	0.000	1.496	0.946	1.472	1.305	0.254	**0.002**
20:5/16:4 (*m/z* 791) MGDG	0.000	0.000	0.000	0.000	0.000	15.378	16.055	14.399	15.277	0.679	**<0.001**
20:5/16:3 (*m/z* 793) MGDG	0.000	0.000	0.000	0.000	0.000	19.508	24.580	25.059	23.049	2.511	**<0.001**
20:5/16:2 (*m/z* 795) MGDG	0.000	0.000	0.000	0.000	0.000	6.150	5.392	5.028	5.523	0.467	**<0.001**
20:4/16:3 (*m/z* 795) MGDG	0.000	0.000	0.000	0.000	0.000	1.573	1.379	1.286	1.412	0.120	**<0.001**
20:3/16:4 (*m/z* 795) MGDG	0.000	0.000	0.000	0.000	0.000	0.208	0.182	0.170	0.187	0.016	**<0.001**
20:5/16:1 (*m/z* 797) MGDG	0.000	0.000	0.000	0.000	0.000	2.536	3.681	1.486	2.567	0.896	**0.015**
20:4/16:2 (*m/z* 797) MGDG	0.000	0.000	0.000	0.000	0.000	0.144	0.209	0.084	0.145	0.051	**0.015**
18:3/18:3 (*m/z* 797) MGDG	4.356	3.789	2.480	3.542	0.786	0.000	0.000	0.000	0.000	0.000	**0.003**
20:5/16:0 (*m/z* 799) MGDG	0.000	0.000	0.000	0.000	0.000	2.863	2.676	2.154	2.564	0.300	**<0.001**
20:4/16:1 (*m/z* 799) MGDG	0.000	0.000	0.000	0.000	0.000	0.241	0.225	0.181	0.216	0.025	**<0.001**
18:3/18:2 (*m/z* 799) MGDG	2.289	4.667	3.657	3.538	0.975	0.000	0.000	0.000	0.000	0.000	**0.007**
18:3/18:1 (*m/z* 801) MGDG	0.974	1.057	1.578	1.203	0.268	0.000	0.000	0.000	0.000	0.000	**0.003**
18:2/18:2 (*m/z* 801) MGDG	1.771	1.923	2.870	2.188	0.486	0.000	0.000	0.000	0.000	0.000	**0.003**
14:0/16:1 (*m/z* 885) DGDG	0.000	0.000	0.000	0.000	0.000	2.494	1.528	1.212	1.745	0.545	**0.011**
18:3/14:0 (*m/z* 909) DGDG	2.015	0.940	2.125	1.693	0.535	0.000	0.000	0.000	0.000	0.000	**0.011**
16:1/16:2 (*m/z* 909) DGDG	0.650	0.303	0.686	0.546	0.172	0.000	0.000	0.000	0.000	0.000	**0.011**
16:0/16:3 (*m/z* 909) DGDG	0.370	0.172	0.390	0.311	0.098	0.000	0.000	0.000	0.000	0.000	**0.011**
18:2/14:0 (*m/z* 911) DGDG	1.143	0.454	3.308	1.635	1.216	0.000	0.000	0.000	0.000	0.000	0.13
16:1/16:1 (*m/z* 911) DGDG	0.156	0.062	0.451	0.223	0.166	6.803	4.028	6.491	5.774	1.241	**0.003**
16:0/16:2 (*m/z* 911) DGDG	0.646	0.257	1.869	0.924	0.687	0.000	0.000	0.000	0.000	0.000	0.13
16:1/16:0 (*m/z* 913) DGDG	0.000	0.000	0.000	0.000	0.000	6.129	4.688	8.445	6.421	1.548	**0.004**
14:0/18:1 (*m/z* 913) DGDG	0.000	0.000	0.000	0.000	0.000	0.688	0.526	0.948	0.721	0.174	**0.004**
18:3/16:2 (*m/z* 933) DGDG	2.688	3.165	1.877	2.577	0.532	0.000	0.000	0.000	0.000	0.000	**0.002**
18:2/16:3 (*m/z* 933) DGDG	1.137	1.338	0.794	1.090	0.225	0.000	0.000	0.000	0.000	0.000	**0.002**
18:3/16:1 (*m/z* 935) DGDG	0.820	0.755	0.970	0.848	0.090	0.000	0.000	0.000	0.000	0.000	**<0.001**
18:2/16:2 (*m/z* 935) DGDG	3.778	3.481	4.470	3.910	0.414	0.000	0.000	0.000	0.000	0.000	**<0.001**
18:3/16:0 (*m/z* 937) DGDG	2.896	0.699	2.933	2.176	1.044	0.000	0.000	0.000	0.000	0.000	**0.042**
18:2/16:1 (*m/z* 937) DGDG	1.324	0.320	1.341	0.995	0.478	0.000	0.000	0.000	0.000	0.000	**0.042**
18:1/16:2 (*m/z* 937) DGDG	0.423	0.102	0.428	0.318	0.152	0.000	0.000	0.000	0.000	0.000	**0.042**
16:1/18:1 (*m/z* 939) DGDG	0.000	0.000	0.000	0.000	0.000	1.557	1.425	0.423	1.135	0.506	**0.034**
18:2/16:0 (*m/z* 939) DGDG	8.349	4.960	6.121	6.477	1.406	0.000	0.000	0.000	0.000	0.000	**0.003**
18:1/16:0 (*m/z* 941) DGDG	2.122	1.072	3.733	2.309	1.095	0.000	0.000	0.000	0.000	0.000	**0.041**
20:5/16:2 (*m/z* 957) DGDG	0.000	0.000	0.000	0.000	0.000	1.434	1.304	0.265	1.001	0.523	0.054
20:5/16:1 (*m/z* 959) DGDG	0.000	0.000	0.000	0.000	0.000	5.519	3.086	2.435	3.680	1.327	**0.017**
20:5/16:0 (*m/z* 961) DGDG	0.000	0.000	0.000	0.000	0.000	2.339	2.124	3.224	2.562	0.476	**0.002**
20:4/16:1 (*m/z* 961) DGDG	0.000	0.000	0.000	0.000	0.000	0.238	0.216	0.328	0.260	0.048	**0.002**

APPENDIX II

Statistics of *H. ostrearia* galactolipids at 20° and 30°C

Relative percent abundances for each replicate in each treatment of *H. ostrearia*, along with averages, standard deviations, and p-values (significant differences in bold)

Galactolipid Species	Replicate 1 20° C	Replicate 2 20° C	Replicate 3 20° C	Mean 20° C	Standard Deviation 20° C	Replicate 1 30° C	Replicate 2 30° C	Replicate 3 30° C	Mean 30° C	Standard Deviation 30° C	P-value
16:3/16:3 (*m/z* 741) MGDG	0.000	0.000	0.000	0.000	0.000	4.762	1.966	0.116	2.281	1.910	0.166
16:1/16:2 (*m/z* 747) MGDG	0.000	0.000	0.000	0.000	0.000	1.043	0.770	0.888	0.901	0.112	**<0.001**
16:0/16:3 (*m/z* 747) MGDG	0.000	0.000	0.000	0.000	0.000	1.418	1.048	1.208	1.225	0.152	**<0.001**
16:1/16:0 (*m/z* 751) MGDG	0.000	0.000	0.000	0.000	0.000	4.248	1.888	0.735	2.291	1.462	0.091
18:3/16:3 (*m/z* 769) MGDG	8.664	14.352	12.623	11.880	2.381	32.996	43.951	40.297	39.081	4.554	**0.002**
18:3/16:2 (*m/z* 771) MGDG	12.108	10.759	14.492	12.453	1.543	9.289	3.459	9.355	7.368	2.764	0.086
18:2/16:3 (*m/z* 771) MGDG	5.011	4.453	5.998	5.154	0.639	4.690	1.746	4.723	3.720	1.395	0.257
18:4/16:0 (*m/z* 773) MGDG	0.000	0.000	0.000	0.000	0.000	0.750	0.312	1.148	0.736	0.341	**0.038**
18:3/16:1 (*m/z* 773) MGDG	3.112	3.482	2.762	3.119	0.294	2.471	1.026	3.779	2.425	1.124	0.446
18:2/16:2 (*m/z* 773) MGDG	23.724	26.548	21.061	23.777	2.240	2.343	0.973	3.584	2.300	1.066	**<0.001**
18:1/16:3 (*m/z* 773) MGDG	0.000	0.000	0.000	0.000	0.000	0.764	0.317	1.169	0.750	0.348	**0.038**
18:3/16:0 (*m/z* 775) MGDG	1.469	1.740	1.477	1.562	0.126	0.000	0.000	0.000	0.000	0.000	**<0.001**
18:2/16:1 (*m/z* 775) MGDG	2.844	3.370	2.860	3.025	0.244	0.000	0.000	0.000	0.000	0.000	**<0.001**
18:1/16:2 (*m/z* 775) MGDG	1.181	1.399	1.187	1.256	0.101	0.000	0.000	0.000	0.000	0.000	**<0.001**
18:2/16:0 (*m/z* 777) MGDG	3.693	3.891	2.092	3.225	0.805	0.000	0.000	0.000	0.000	0.000	**0.005**
18:1/16:1 (*m/z* 777) MGDG	0.532	0.561	0.301	0.465	0.116	0.000	0.000	0.000	0.000	0.000	**0.005**
18:0/16:2 (*m/z* 777) MGDG	0.077	0.081	0.043	0.067	0.017	0.000	0.000	0.000	0.000	0.000	**0.005**
18:1/16:0 (*m/z* 779) MGDG	1.021	1.762	0.503	1.096	0.245	0.000	0.000	0.000	0.000	0.000	**0.04**
16:1/18:0 (*m/z* 779) MGDG	0.220	0.381	0.109	0.237	0.383	0.000	0.000	0.000	0.000	0.000	**0.04**
20:5/16:3 (*m/z* 793) MGDG	0.000	0.000	0.000	0.000	0.000	16.077	17.676	6.195	13.316	5.078	**0.021**
20:5/16:2 (*m/z* 795) MGDG	0.000	0.000	0.000	0.000	0.000	4.531	3.604	2.628	3.587	0.777	**0.003**
20:4/16:3 (*m/z* 795) MGDG	0.000	0.000	0.000	0.000	0.000	0.679	0.540	0.394	0.538	0.116	**0.003**
20:5/16:1 (*m/z* 797) MGDG	0.000	0.000	0.000	0.000	0.000	2.186	2.482	1.297	1.988	0.503	**0.005**
18:3/18:3 (*m/z* 797) MGDG	4.753	3.347	9.122	5.741	2.459	5.968	6.776	3.541	5.428	1.375	0.883
18:3/18:2 (*m/z* 799) MGDG	3.424	3.747	4.398	3.856	0.405	0.000	0.000	0.000	0.000	0.000	**<0.001**
18:3/18:1 (*m/z* 801) MGDG	0.384	0.315	0.188	0.296	0.081	0.000	0.000	0.000	0.000	0.000	**0.007**
18:2/18:2 (*m/z* 801) MGDG	1.223	1.002	0.599	0.941	0.258	0.000	0.000	0.000	0.000	0.000	**0.007**
18:3/16:3 (*m/z* 931) DGDG	5.018	1.677	1.238	2.644	1.688	0.000	0.000	0.000	0.000	0.000	0.091
20:5/14:0 (*m/z* 933) DGDG	0.000	0.000	0.000	0.000	0.000	0.010	0.099	0.442	0.184	0.186	0.235
18:3/16:2 (*m/z* 933) DGDG	1.659	3.428	1.559	2.215	0.859	0.092	0.929	4.130	1.717	1.741	0.735
18:2/16:3 (*m/z* 933) DGDG	0.911	1.882	0.856	1.216	0.471	0.000	0.000	0.000	0.000	0.000	**0.022**
16:3/18:2 (*m/z* 933) DGDG	0.000	0.000	0.000	0.000	0.000	0.068	0.686	3.051	1.268	1.285	0.235
18:3/16:1 (*m/z* 935) DGDG	0.391	0.461	0.665	0.506	0.116	0.017	1.049	1.501	0.856	0.621	0.477
18:2/16:2 (*m/z* 935) DGDG	2.188	2.577	3.718	2.828	0.649	0.035	2.139	3.062	1.745	1.267	0.343
18:3/16:0 (*m/z* 937) DGDG	3.237	0.671	0.485	1.464	1.256	1.244	0.203	1.021	0.823	0.447	0.533
18:2/16:1 (*m/z* 937) DGDG	2.222	0.461	0.333	1.005	0.862	0.000	0.000	0.000	0.000	0.000	0.174
18:1/16:2 (*m/z* 937) DGDG	0.318	0.066	0.048	0.144	0.123	0.000	0.000	0.000	0.000	0.000	0.174
18:2/16:0 (*m/z* 939) DGDG	6.433	2.763	8.272	5.823	2.290	1.524	2.105	2.218	1.949	0.304	0.077
18:1/16:0 (*m/z* 941) DGDG	0.000	0.000	0.000	0.000	0.000	0.000	0.482	2.998	1.160	1.315	0.28
20:5/16:1 (*m/z* 959) DGDG	0.000	0.000	0.000	0.000	0.000	0.377	0.509	0.070	0.319	0.184	0.07
18:3/18:3 (*m/z* 959) DGDG	2.847	1.489	1.531	1.956	0.630	2.418	3.265	0.449	2.044	1.179	0.93
18:3/18:1 (*m/z* 963) DGDG	0.164	0.410	0.182	0.252	0.112	0.000	0.000	0.000	0.000	0.000	**0.033**
18:2/18:2 (*m/z* 963) DGDG	1.172	2.926	1.296	1.798	0.799	0.000	0.000	0.000	0.000	0.000	**0.033**

CHAPTER III

NOW YOU SEE IT, NOW YOU DON'T: DIFFERENCES IN HYDROCARBON PRODUCTION IN THE DIATOM *PHAEODACTYLUM TRICORNUTUM* DUE TO GROWTH TEMPERATURE

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INTRODUCTION

Studies on algal lipid production, with particular emphasis on microalgae, have risen in importance due to the growing search for a cheap, sustainable, and easy-to-grow biological source of biofuels (see review by Mata et al., 2010). There are a number of advantages to microalgal oil, which can contain hydrocarbons (see below for further discussion), isoprenoids, triglycerides (TAGs), or fatty acid methyl esters (FAMEs) (Dismukes et al. 2008; Gong & Jiang, 2011), when compared with oil from higher plants: the percentage of oil produced is greater per unit weight, the amount of land needed to cultivate large amounts of biomass is smaller, its production does not compete for space with food crops, and the doubling rate of the organisms can be on the scale of hours to days as opposed to the months it takes to produce a crop (Gong & Jiang, 2011). However, current biofuel sources such as ethanol, which is not a lipid but a microbial fermentation product produced from sugars and polysaccharides, and biodiesel, which usually consists of FAMEs in the C_{12} to C_{22} range generally produced from vegetable oil or animal fat, face numerous hurdles before they can be accepted as alternatives to petroleum-based fuels. For example, ethanol by itself is not compatible with the current fuel infrastructure as its ability to absorb water often corrodes engines and pipes, and its energy content is only 70% of that of gasoline (Lee et al., 2008; Peralta-Yahya et al., 2012). Biodiesel, likewise, has a lower energy content (around 11% lower) than does petroleum-derived diesel, possibly due to the FAMEs being already partially oxidized, and also gels at colder temperatures if made from saturated fats, while high amounts of unsaturated fats are susceptible to oxidation and have problems with long-term storage (Lee et al., 2008; Hu et

al. 2008). In the short term, biological products that are closer in composition to currently used fossil fuels are the ideal targets for the search of a sustainable fuel source.

It has been hypothesized that, due the growing number of genetic tools available, biofuel companies and researchers may be able to "milk" diatoms for oil or even have them produce some components of gasoline (Radakovits et al., 2010; Ramachandra et al., 2009). Diatoms are one of the largest and most diverse groups of primary producers on the planet, numbering in the 100,000s of species (Moustafaet al., 2009; Seckbach & Kociolek, 2011). As a group, diatoms are responsible for nearly half of marine primary production, and a quarter of the planet's total primary production (Werner, 1977). Diatoms are known to produce high amounts of lipids, anywhere from 50%-60% of their biomass weight, with about 75% of those lipids being neutral lipids such as TAGs, isoprenoids, and hydrocarbons (Ramachandra et al., 2009). Hydrocarbons have been characterized in diatoms previously, with a large number of studies being performed on the ability of diatoms, particularly *Haslea ostrearia* and other members of its genus, to produce highly branched isoprenoids (HBIs) with carbon numbers typically ranging from C_{20} to C_{30} (Allard et al., 2001; Belt et al., 2006; Rowland et al., 2001; Volkman et al., 1994). The diatoms *Pleurosigma intermedium, Rhizoselenia setigera, Navicula sclesvicensis,* and the recent discovery of HBIs in the diatom *Berkeleya rutilans* have also been found to produce different HBIs (Grossi et al., 2004; Belt et al., 2001; Sinninghe Damsté et al., 2000; Brown et al., 2014, respectively). The biological purpose of these HBIs is currently unknown, although it has been shown that the unsaturation of the HBIs increases with increasing growth temperature (Rowland et al., 2001), contrary to what would normally be expected during homeoviscous adaptation of membrane lipids like

phospholipids and galactolipids, and they have been primarily examined for their possible pharmacological applications and presence in sediments as biomarkers of past diatom blooms (Rowland & Robson, 1990). Smaller isoprenoids, in the C_{10} to C_{15} range, such as farnesane and bisabolane which have been produced by genetically engineered yeast and bacteria respectively, have the potential to become a source of biofuel, as their branches, double bonds, and ring structures satisfy the need for molecules with fluidity at freezing cold as isoprenoid-derived biofuels are being considered for use as jet fuels (Lee et al., 2008; Peralta-Yahya et al., 2012); however, these same features also cause a decrease in combustion quality (Lee et al., 2008; Peralta-Yahya et al., 2012).

Alkanes are saturated hydrocarbons containing only carbon and hydrogen atoms, can be found in linear or branched forms, and are a major component of commonly used fuels such as gasoline and diesel. Gasoline contains C_4 to C_{12} hydrocarbons, diesel fuel contains C_9 to C_{23} hydrocarbons, and jet fuel contains C_8 to C_{16} hydrocarbons, all of which can be linear, branched, and/or cyclical (Lee et al., 2008). Although algae have not yet become a commercial source of hydrocarbon fuel, there are a number of promising prospects. For example, the chlorophyte, *Botryococcus braunii*, was found to produce very long-chain hydrocarbons, ranging from C_{23} to C_{33} dienes and trienes in one strain and C_{30} to C_{37} triterpenoids in another (Metzger & Largeau, 2005). The oils from *B. braunii*, particularly the triterpene botryococcenes, are unable to be used as biofuel directly due to their inability to be transesterified since they lack the necessary carboxylic acid functional group, but can be used as a feedstock for hydrocracking in oil refineries to produce octane (C_8H_{18}), kerosene, and diesel (Hillen et al., 1982). Since long-chain hydrocarbons and highly-branched hydrocarbons must be further processed before they are usable as a fuel

source, either because they cannot be transesterified or the long carbon chains cause them to gel at freezing temperatures, examining organisms for their ability to produce shorter hydrocarbons (from C_4 to C_{23}) may provide a step towards an answer since they can be used directly as a fuel source (Radakovits et al., 2010).

Outside of HBIs, alkane and alkene production in diatoms is not well characterized. McKay et al (1996) studied the release of hydrocarbons as gasses from the centric diatom *Skeletonema costatum* and the dinoflagellate *Scrippsiella trochoidea*, and found that the diatom did release hydrocarbons ranging from ethane (C_2H_6) to octane (C_8H_{18}) in gaseous form as well as some alkenes such as propene ($C_{3:1}$) and butene ($C_{4:1}$). They also saw that the amount of hydrocarbons produced increased as the cultures aged. *R. setigera* was also found to produce linear polyunsaturated alkenes that ranged from C_{21} to C_{27} with relative percentages as low as 1% and as high as 37% depending on growth temperature, as well as small amounts, between 1% and 4% relative abundance, of heptadecane (Sinninghe Damsté et al., 2000). Sinninghe Damsté et al. (2000) hypothesize that production of these alkenes may be related to buoyancy and vertical migration, and they state that temperature has no effect on the ratio of the alkene isomers they discovered.

Phaeodactylum tricornutum is a model pennate diatom, being one of a few diatoms to have its complete genome sequenced, and has been successfully transformed with genes coding for antibiotic resistance and other reportable genes (Apt et al., 1996; Bowler et al., 2008, Zaslavskaia et al., 2001). *P. tricornutum* is common in both coastal and inland bodies of water, usually in areas that provide unstable, in terms of both temperature and nutrient content/quality, living environments, such as estuaries and tidal pools, indicating its ability to acclimate to stressful conditions (Rushforth et al., 1988). Lipid accumulation,

namely accumulation of TAGs, has been studied in *P. tricornutum* under a number of stressful growth conditions, such as nitrogen depletion, and it has been a model for lipid metabolism studies as well (Hu et al., 2008; Valenzuela et al., 2012). To our knowledge, no studies of short- to medium-chain hydrocarbon production in *P. tricornutum* have been performed. To this end, we have performed an initial survey into the potential of *P. tricornutum* to produce short- or medium-chain hydrocarbons (those in the C_4 to C_{23} range seen in petroleum-based fuels), and if it does possess this ability, to examine whether growth temperature affects hydrocarbon production in a similar manner to what we observed in the modulation of galactolipid-associated fatty acids (see below).

Previously, we have examined the effects of growth temperature on galactolipids, the primary component of chloroplast membranes and lipids that are not known to be used as energy storage, of the pennate diatoms *H. ostrearia* and *P. tricornutum* (Dodson et al., 2014). At 20°C, both *P. tricornutum* and *H.* ostrearia possessed different galactolipid profiles, with the former possessing mostly C_{20}/C_{16} (*sn*-1/*sn*-2) forms of mono- and digalactosyldiacylglycerol (MGDG and DGDG, respectively) and the latter possessing mostly C_{18}/C_{16} forms of MGDG and DGDG. We discovered, however, that both diatoms had similar lipid profiles when grown at a 30°C: more saturated lipids, an increase in C_{18} fatty acids at the *sn*-1 position of the lipid, and a complete lack of any C_{20} fatty acids. The specific objectives of this study on hydrocarbon production by *P. tricornutum* were therefore twofold: 1) to identify the hydrocarbons produced by *P. tricornutum* under the optimal growth temperature of 20°C (Bojko et al., 2013), and 2) to examine how a higher, more stressful growth temperature affects the hydrocarbons produced. In order to do this, lipid extracts from *P. tricornutum* cultures grown at 20° and 30°C were analyzed by gas

chromatography-chemical ionization/mass spectrometry (GC-CI/MS). Our study has demonstrated that *P. tricornutum* produced a number of hydrocarbons, including undecane, when grown at 20°C. However, when grown at 30°C, the hydrocarbon profile changed dramatically, with a very different set of hydrocarbons appearing in place of those seen at the lower temperature. Based on what was seen in this study, the types of hydrocarbons produced, and hence fuel quality, may be greatly affected by the growth temperature of this particular model organism.

MATERIALS AND METHODS

Cultures and growth conditions

Phaeodactylum tricornutum Bohlin CCMP 1327 was obtained from the National Center for Marine Algae and Microbiota (NCMA, East Boothbay, Maine, USA). Two sets of acclimated cultures were grown in triplicate in 2L of L1 medium to late exponential phase in separate incubators at 20°C and 30°C, an irradiance of ~50 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}, and a light:dark cycle of 14:10 hours; they were harvested according to the methodology of Leblond & Chapman (2000).

Lipid extraction and fractionation

Total lipids were extracted, and hydrocarbons were separated from other lipid classes according to the techniques described by Leblond and Chapman (2000). Briefly, the total lipid extracts were separated into five component lipid fractions on columns of activated Unisil silica (1.0 g, 100–200 mesh, activated at 120C; Clarkson Chromatography, South Williamsport, PA, USA). The following solvent regime was used to separate lipids according to polarity, with fraction 5 eluting the most polar lipids

(Leblond and Chapman 2000): (1) 12 mL methylene chloride (hydrocarbons and sterol esters), (2) 15 mL 5% acetone in methylene chloride with 0.05% acetic acid (free sterols, tri- and diacylglycerols, and free fatty acids), (3) 10 mL 20% acetone in methylene chloride (monoacylglycerols), (4) 45 mL acetone (chloroplast glycolipids) and (5) 15 mL methanol with 0.1% acetic acid (polar lipids, including phosphorous- and non-phosphorous-containing lipids).

Mass spectrometry of lipids

Lipid extracts from the first fraction were subjected to GC-CI/MS. GC-CI/MS analysis was performed on a Thermo Trace GC Ultra Gas Chromatograph coupled to a TSQ Quantum XLS GC-MS (Thermo Fisher Scientific, Waltham, MA, USA) using an RTX-5MS capillary column (30 m length, 0.25 mm inner diameter, 0.25 μm film 379 thickness, Restek, Bellefonte, PA, USA) with the following GC-CI/MS conditions: splitless injection with injector set at 280°C, a scan range of 50-550 amu and scan time of 0.2 sec, column held at 50°C for 0.5 min, increased to 170°C at 10°C per min, increased to 210°C at 2°C per min and held for 10 min, then increased to 300°C at 5°C per min with a final hold for 7 min. The transfer line was set at 275°C, and helium was delivered at a constant velocity of 40 cm per sec. The emission current was set to 25 μA and the methane flow for chemical ionization was set to 0.1 mL per min. A purchased mixture of alkane standards from octane to eicosane (Sigma-Aldrich, St. Louis, MO, USA) and a tricosane standard (Eastman Chemical, Kingsport, TN, USA), were used to calculate Kovats retention indices (RI) for peaks of interest and to confirm identification of *P. tricornutum*-produced hydrocarbons, where possible. Detailed GC/MS information, such as retention times (RT), retention indices (RI), and *m/*z of the standards can be seen in

Table 1. Peak area was measured for each hydrocarbon in the chromatogram in order to calculate average relative percent abundances for each temperature treatment. Standard deviations were calculated for each triplicate set.

RESULTS

Detailed information - including retention time (RT), retention index (RI), *m/z*, average relative percent abundance, and standard deviations - about the hydrocarbons found in *P. tricornutum* at both growth temperatures can be found in Table 2, and identifications as based on hydrocarbon mass spectra are discussed below. *P. tricornutum* produced a variety of hydrocarbons at 20°C. One standout hydrocarbon, due to its potential ability to be used in a replacement of any petroleum-based fuel, was that of undecane ($C_{11}H_{25}$, *m/z* 156) at RT 7.78 min and relative abundance of 5.3% ± 2.8, whose calculated RI of 1102 was very close to the expected RI of undecane, 1100. Other alkanes detected include octane (C_8H_{18}, *m/z* 114, RT 3.75 min) at a relative abundance of 6.1% ± 2.8 appeared with an RI of 802, which was close to the expected RI of octane at 800, nonadecane ($C_{19}H_{40}$, *m/z* 268, RT 22.41 min) at a and relative abundance of 20.0% ± 6.4 with an RI of 1917, close to the expected RI of 1900, and heneicosane ($C_{21}H_{44}$, *m/z* 296, RT 28.53 min) at a relative abundance of 2.4% ± 0.9 with an RI of 2100, which matched the expected RI. There were two peaks that were unable to be fully identified in the 20°C chromatogram: the most abundant molecule, at 43.9% ± 6.6 relative abundance (RT 27.38 min, RI 2067) and another molecule at 22.3% ± 4.8 relative abundance (RT 35.21 min, RI 2291. Parent masses for these two compounds could not be identified; however, their

Table 1. Identification and details of hydrocarbon standards			
Name, formula	Retention Time	*m/z*	RI
octane, C_8H_{18}	3.73	114	800
nonane, C_9H_{20}	4.58	128	900
decane, $C_{10}H_{22}$	6.31	142	1000
undecane, $C_{11}H_{24}$	7.78	156	1100
dodecane, $C_{12}H_{26}$	9.25	170	1200
tridecane, $C_{13}H_{28}$	10.67	184	1300
tetradecane, $C_{14}H_{30}$	12.02	198	1400
pentadecane, $C_{15}H_{32}$	13.36	212	1500
hexadecane, $C_{16}H_{34}$	14.93	226	1600
heptadecane, $C_{17}H_{36}$	16.85	240	1700
octadecane, $C_{18}H_{38}$	19.19	254	1800
nonadecane, $C_{19}H_{40}$	21.90	268	1900
eicosane, $C_{20}H_{42}$	25.06	282	2000
tricosane, $C_{23}H_{48}$	35.50	324	2300

Table 2. Identification and details of hydrocarbons found in *P. tricornutum* at 20°C and 30°C.

Retention time at 20°C (min)	Retention time at 30°C (min)	Identification	*m/z*	RI at 20°C	RI at 30°C	Average relative % abundance at 20°C	Standard Deviation of % relative abundance at 20°C	Average relative % abundance at 30°C	Standard Deviation of % relative abundance at 30°C
3.75		octane, C_8H_{18}	114	802		6.1	2.8		
7.78		undecane, $C_{11}H_{25}$[a]	156	1102		5.3	2.8		
	16.36	heptadecene, $C_{17}H_{34}$	238		1674			10.3	1.8
	16.95	heptadecane, $C_{17}H_{36}$[b]	240		1706			44.1	3.5
	18.83	octadecene, $C_{18}H_{36}$	252		1785			1.7	0.5
	19.03	octadecane, $C_{18}H_{38}$	254		1793			6.0	1.6
	20.13	Unidentified[d]	Unkn		1835			10.3	2.4
	20.76	Unidentified	Unkn		1858			3.3	1.2
	21.04	nonadecene, $C_{19}H_{38}$	266		1868			2.8	0.9
	21.35	Unidentified	Unkn		1880			8.9	1.8
22.41	21.85	nonadecane, $C_{19}H_{40}$	268	1918	1898	20.0	6.4	9.9	2.4
27.38		Unidentified[c]	Unkn	2067		43.9	6.6		
28.53		heneicosane, $C_{21}H_{44}$	296	2100		2.4	0.9		
	29.45	eicosane, $C_{20}H_{42}$	282		2126			2.8	2.0
35.21		Unidentified	Unkn	2292		22.3	4.8		

[a]Spectra shown in Fig. 1a
[b]Spectra shown in Fig. 1c
[c]Spectra shown in Fig. 2a
[d]Spectra shown in Fig. 2b

mass spectra were similar to previously published data on diatom-produced HBIs (see discussion below).

At 30°C, *P. tricornutum* produced a larger variety of hydrocarbons, including a number of alkanes and alkenes, however many of the hydrocarbons produced were not seen at the lower temperature. The most prominent peak, at 44.1% ± 3.5 relative abundance, was at RT 16.95 min and was identified as heptadecane ($C_{17}H_{36}$, *m/z* 240) with an RI of 1705, which was very close to the expected RI of 1700. Other alkanes produced were octadecane ($C_{18}H_{40}$, *m/z* 254, RT 19.03 min, RI 1793) at 6.0% ± 1.6 relative abundance, nonadecane ($C_{19}H_{40}$, *m/z* 268, RT 21.85 min, RI 1898) at 9.9% ± 0.9 relative abundance, and eicosane ($C_{20}H_{42}$, *m/z* 282, RT 29.45, min, RI 2126) at 2.8% ± 2.0 relative abundance. Interestingly, at 30°C there were also three alkenes detected, corresponding to the three shortest alkanes identified at 30°C: heptadecene ($C_{17}H_{34}$, *m/z* 238, RT 16.36 min, RI 1674) at 10.3% ± 1.8 relative abundance, octadecene ($C_{18}H_{36}$, *m/z* 252, RT 18.83 min, RI 1785) at 1.7% ± 0.5 relative abundance, and nonadecene ($C_{19}H_{38}$, *m/z* 266, RT 21.04 min, RI 1868) at 2.8% ± 0.9 relative abundance. All three alkenes detected were at a lower relative percent abundance than their corresponding alkanes. There were also three unidentified molecules that all had similar spectra: one at 10.3% ± 2.4 relative abundance (RT 20.13 min, RI 1835), one at 3.3% ± 1.2 relative abundance (RT 20.76 min, RI 1858), and another at 8.9% ± 1.8 relative abundance (RT 21.35 min, RI 1868) for which parent ions could not be identified.

Figure 1 shows the mass spectra of four compounds as examples of identification of alkanes, one alkane detected from *P. tricorntutum* at 20°C (undecane, $C_{11}H_{24}$), one alkane detected from *P. tricornutum* at 30°C (heptadecane, $C_{17}H_{36}$, *m/z*), and the

standards that were used to properly identify them. The most prominent characteristic of alkane fragmentation is the 14 *m/z* loss from each of the prominent peaks in the spectra corresponding to the loss of a CH_2 group. The parent ions are also present at 156 *m/z* for undecane ($C_{11}H_{24}$) and 240 *m/z* for heptadecane ($C_{17}H_{36}$). Figure 2 shows one of the unidentified molecules from each temperature. Both unidentified compounds, for example in Figure 2a, from *P. tricornutum* at 20°C share similar fragmentation patterns (prominent peaks at 67, 79, and 91 *m/z*), but parent ions were not readily apparent and the 12 *m/z* loss from each peak is most likely indicative of double bonds (loss of a single carbon). Similarly, the three unidentified compounds, for example in Figure 2b, seen at 30°C also share similar fragmentation patterns (prominent peaks at 55, 68, 82, and 95 *m/z*), but again parent ions cannot be seen. All of these compounds eluted from fraction 1 and therefore must be very non-polar.

DISCUSSION

Inspired by the thought that genetic engineering of microalgae could eventually lead to the sustainable production of biofuels that require little to no processing, we have examined the hydrocarbon lipid fraction of *P. tricornutum*. *P. tricornutum* has been used as the model diatom for studies on growth conditions, lipid accumulation, and diatom genetics and genomics (Bowler et al., 2008; Hu et al., 2008; Valenzuela et al., 2012). *P. tricornutum* has also been successfully transformed with genes coding for antibiotic resistance, and genes have successfully been cloned from *P. tricornutum* into yeast in order to facilitate EPA biosynthesis (Apt et al., 1996; Domergue et al., 2002, Zaslavskaia

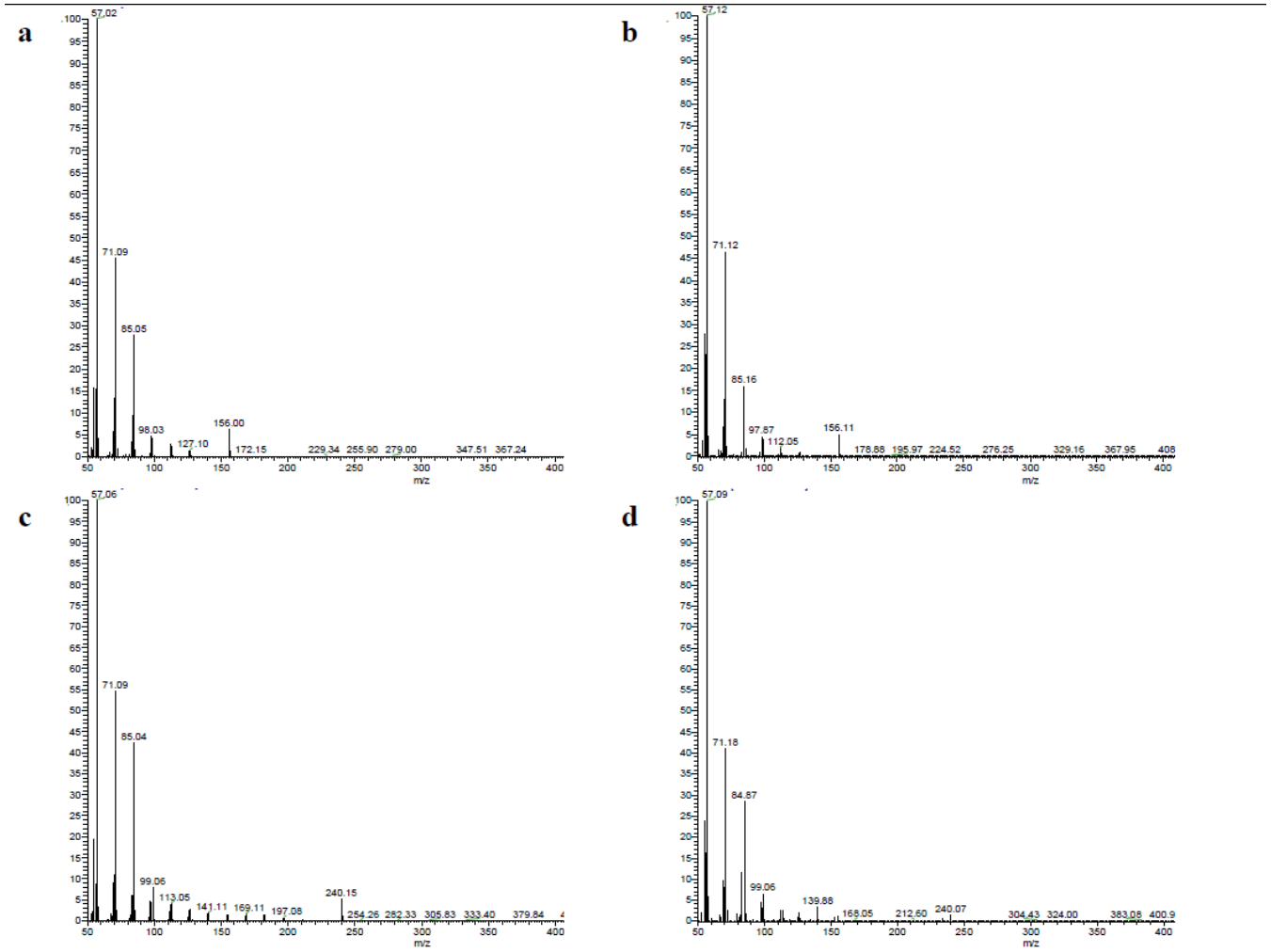

Fig. 1 Comparison of GC-MS spectra of (a) undecane from *P. tricornutum* grown at 20°C to (b) the undecane in the mixed alkane standard; and (c) heptadecane from *P. tricornutum* grown at 20°C to (d) the heptadecane in the mixed alkane standard

et al., 2001). Due to its potential to be used as a model for engineering of diatoms to produce biofuels, we felt that examining *P. tricornutum*'s potential to produce hydrocarbons, such as those found in current petroleum-based fuels, would give us an idea of the inherent capability of this organism for that purpose. It is important to point out that *P. tricornutum* did in fact produce hydrocarbons similar in size to those found in petroleum fuels: C_4 to C_{12} for gasoline, C_9 to C_{23} for diesel fuel, and C_8 to C_{16} for jet fuel. The alkanes produced made up 34% and 76% of the total hydrocarbons produced at 20°C and 30°C, respectively, indicating that temperature stress also has an effect on lipid production, and invoking the thought that perhaps *P. tricornutum* can be engineered and manipulated into producing these hydrocarbons in greater quantities. The hydrocarbons produced, however, differed between the two temperature treatments, yet octadecane ($C_{18}H_{38}$) was present at both temperatures and found not to be significantly different between the two treatments with a p-value of 0.0635. At 20°C, *P. tricornutum* produced two short-chain alkanes, octane (C_8H18, *m/z* 114) and undecane ($C_{11}H_{24}$, *m/z* 156), and two medium-chain alkanes, nonadecane ($C_{19}H_{40}$, *m/z* 268) and heneicosane ($C_{21}H_{44}$m *m/z* 296). When grown at 30°C, however, the shortest chain alkane that was produced was heptadecane ($C_{17}H_{36}$, *m/z* 240) while the longest chain produced was eicosane ($C_{20}H_{42}$, *m/z* 282). *P. tricornutum* also produced three alkenes at the higher growth temperature, and these hydrocarbons were not seen at the lower growth temperature. Similarly to what was seen in the HBIs produced by *H. ostrearia*, unsaturation appears to increase in these hydrocarbons with increased growth temperature (Rowland et al., 2001). The spectra for the two unidentified compounds at 20°C are similar to the mass spectra of the tetra-

unsaturated sesterpenoids produced by *H. ostrearia* as seen by Allard et al. (2001), except that the masses of the fragment ions in the HBI mass spectra in *H. ostrearia* are all 10 mass units smaller than the fragment ions seen in the mass spectra peaks of the unknown compounds from *P. tricornutum.* The retention indices of the peaks from *P. tricornutum* are also close to the same range as the HBIs (RT of 2067 and 2292 in *P. tricornutum* compared to the mid-2100s in *H. ostrearia*; Volkman et al., 1994; Massé et al., 2004). The three unidentified compounds seen at 30°C in *P. tricornutum* are even more similar to the HBIs with the 55, 69, 81, and 95 *m/z* peaks all being present, though two of the most prominent peaks are one mass unit larger or smaller than the expected peaks, such as 68 *m/z* being more prominent than 69 *m/z*, and 82 *m/z* being more prominent than 81*m/z* (Volkman et al., 1994; Massé et al., 2004); this may be due to instrumental variation and/or the use of chemical ionization (CI) in our study versus electron impact ionization (EI) in these other studies. To date, HBIs have only been identified in five diatom genera: *Rhizoselenia, Haslea, Navicula, Pleurosigma,* and very recently *Berkeleya* (Sinninghe Damsté et al., 2004; Brown et al., 2014), and the capability to synthesize these HBIs appears to have evolved separately in two different groups of diatoms (Sinninghe Damsté et al., 2004), one set of pennates (*Berkeleya, Haslea, Pleurosigma,* and *Navicula,* all of them in the order Naviculales like *P. tricornutum*) and a genus of centrics (*Rhizoselenia*). Although *P. tricornutum* was identified by Sinninghe Damsté et al. (2004) as being unable to synthesize HBIs, this conclusion may be premature because only one isolate was examined under a single growth condition. Future studies in our laboratory will examine a range of other isolates of *P. tricornutum* in order to better assess the potential for this

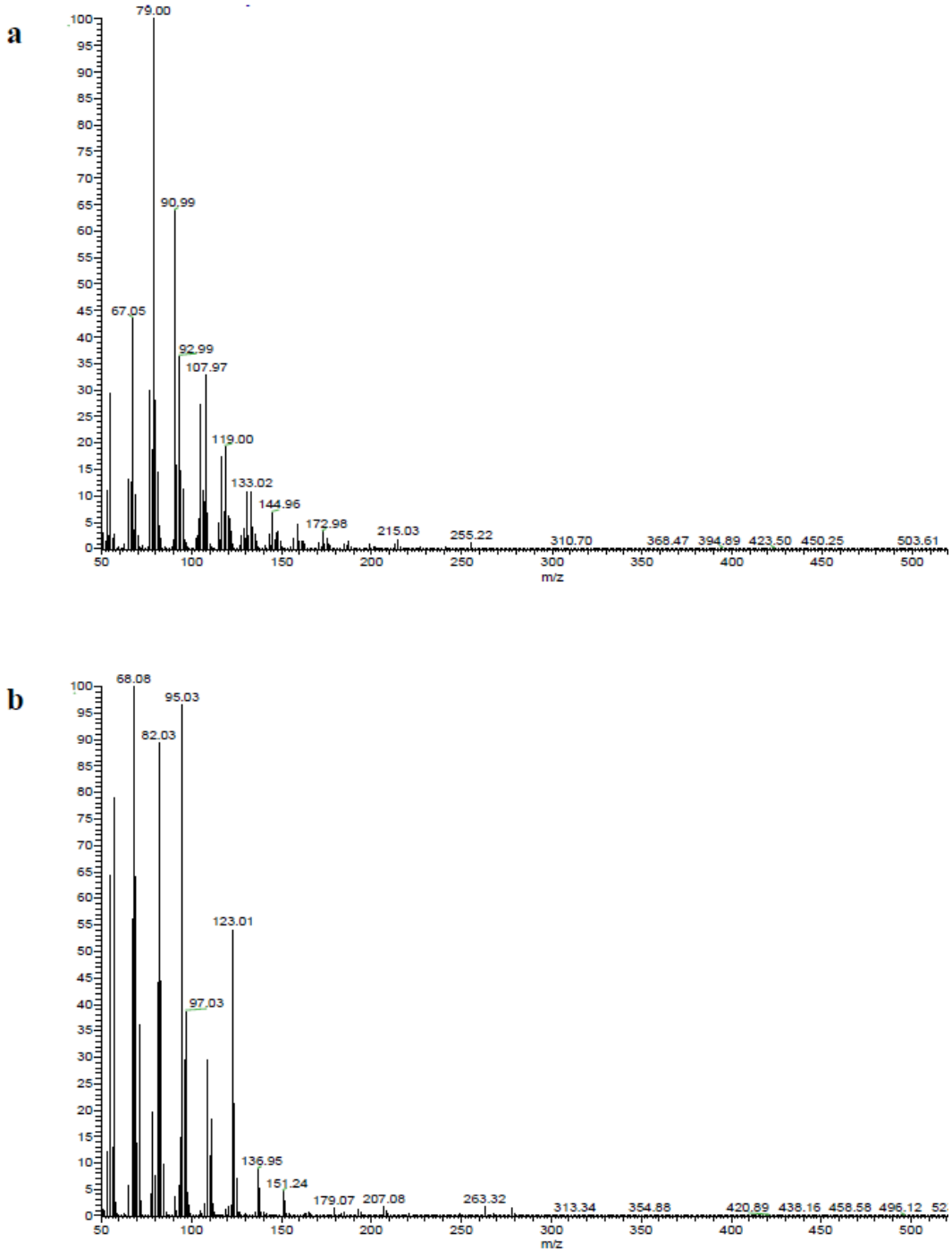

Fig. 2 Examples of unidentified GC-MS spectra in (a) *P. tricornutum* grown at 20°C found at retention time 27.38 min, and (b) *P. tricornutum* grown at 30°C found at retention time 20.13 min

particular model diatom to produce HBIs in addition to the linear hydrocarbons that we have identified.

In our previous study on the chloroplast galactolipids of diatoms at different growth temperatures, we saw a consolidation of fatty acid chain length to 18 carbons in *P. tricornutum* when it was grown at 30°C (Dodson et al., 2014). When grown at 20°C, *P. tricornutum* contained a variety of fatty acids ranging from 14 carbons to 20 carbons at the *sn*-1 position of the galactolipids, whereas these were seemingly replaced by 18 carbon fatty acids at the higher growth temperature. This consolidation is reflected in the hydrocarbons produced by *P. tricorntunum* at the two temperatures, in form as well as magnitude. The most abundant hydrocarbon produced at 20°C, aside from the unidentified compounds, was nonadecane ($C_{19}H_{40}$), while the most abundant hydrocarbon at 30°C was heptadecane ($C_{17}H_{34}$). In studies on TAG synthesis in *P. tricornutum*, it was also found that it produced high amounts of eicosapentaenoic acid (EPA, $C_{20:5}$), as well as palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$), and small amounts of docosahexaenoic acid (DHA, $C_{22:6}$) (Yongmanitchai & Ward, 1993). Fatty acid synthesis in plants and algae has been well studied and is known to take place through one of two different pathways via a type II fatty acid synthase (Mühlroth et al., 2013). Both of these pathways begin in the chloroplast with the production of palmitic acid ($C_{16:0}$) (Blatti et al., 2013). Once palmitic acid is attached to an acyl carrier protein (ACP), it is either moved to the endoplasmic reticulum (ER) for the eukaryotic pathway, or kept inside the chloroplast for the prokaryotic pathway (Dörmann & Benning, 2002). In the eukaryotic pathway, the lipids often possess longer chains and more unsaturations, such as linoleic acid ($C_{18:3}$) or EPA

($C_{20:5}$) (Domergue et al, 2003). In the prokaryotic pathway, the lipids often possess shorter chains, such as 16 and 14 carbons, but can still possess unsaturations, notably $C_{16:3}$ (Domergue et al., 2003). These fatty acids can then be incorporated into membrane lipids or into TAGs for storage (Mühlroth et al., 2013).

In the clonal green chlorophyte *B. braunii*, it was found that alkanes are produced through a three step process involving fatty acids (Dennis & Kolattukudy, 1991). In *B. braunii*, the fatty acids are first activated by coenzyme A. These activated fatty acids are then reduced into aldehydes by an NADH-dependent acyl-CoA reductase, and the aldehydes are then decarbonylated into alkanes by a cobalt-dependent aldehyde decarbonylase. This process produces alkanes that are one carbon shorter than the starting fatty acid, often resulting in an odd number of carbons (Dennis & Kolattukudy, 1991). Therefore, a large number of C_{20} fatty acids would translate to a relatively high percentage nonadecane ($C_{19}H_{40}$). Likewise a large number of C_{18} fatty acids would correspond to a relatively high percentage of heptadecane ($C_{17}H_{36}$). Since we know that *P. tricornutum* produces lots of EPA at 20°C from studies on its TAGs and C_{18} fatty acids at 30°C (Dodson et al., 2014), it makes sense that we detected relatively high amounts of nonadecane ($C_{19}H_{40}$) at 20°C and heptadecane ($C_{17}H_{36}$) at 30°C which result from decarbonylation of C_{20} and C_{18} fatty acids respectively. The presence of relatively small amounts of heneicosane ($C_{21}H_{24}$) at 20°C indicates the production of C_{22} fatty acids by *P. tricornutum* which have been detected in its TAG profile (Hamilton et al., 2014).

The presence of the even-numbered alkanes, such as octane (C_8H_{18}) and octadecane ($C_{18}H_{38}$) is more difficult to explain as these cannot be synthesized through reduction and decarbonylation of even-numbered fatty acids like the odd-numbered

alkanes. Even-numbered alkanes have been seen in *Escherichia coli* strains that were genetically engineered with the *Bacillus subtilis fabH* gene, which codes for β-ketoacyl-ACP synthase which is an acyl transferase and part of the type II fatty acid synthesis pathway seen in plants and bacteria (Choi & Lee, 2013). The possibility exists that the even-numbered alkanes that we detected in our samples are the result of the bacteria associated with the diatom culture as the cultures were not axenic; however, octane (C_8H_{18}) is the most relatively abundant even-numbered hydrocarbon among the two treatments at just 6.1%, while the other even-numbered alkanes, and the single even-numbered alkene, occurred at 6% relative abundance or less. It is also just as possible that these even-numbered are produced by *P. tricornutum* as the centric diatom *S. costatum* was seen to produce and release octane (C_8H_{18}), butane (C_4H_{10}), and hexane (C_6H_{14}), as well as some mono-enes and di-enes (McKay et al., 1996). The centric diatom *R. setigera* was also seen to produce small amounts of heptadecane and some long-chain alkenes between C_{21} and C_{27} (Sinninghe Damsté et al., 2000). In their study on *R. setigera* alkenes, the authors propose that the linear alkenes are synthesized via the elongation, in the form of C_2 units, and subsequent decarboxylation of a $C_{22:6n\text{-}3}$ fatty acid similar to how the alkadienes in *B. braunii* and the hentriacontadienes in the haptophytes are synthesized from octadecenoic acid ($C_{18:1}$; Sinninghe Damsté et al., 2000). One of the alkenes from *R. setigera*, a linear $C_{21:6}$, was also found to be present in a number of other diatoms, including *S. costatum*, two *Thalassiosira* species, *Cyclotella nana, Ditylum brightwellii,* and *Lauderia borealis,* as well as in members of the Dinophyceae, Cryptophyceae, Haptophyceae, and one member of the Euglenophyceae in a study on microalgal hydrocarbons (Blumer et al., 1971). The production of alkanes was also discovered in

strains of the cyanobacteria *Synechocystis, Anabaena,* and *Synechococcus* ranging from C_{15} to C_{20}, including the even-numbered alkanes hexadecane ($C_{16}H_{34}$) and eicosane ($C_{20}H_{42}$) (Tan et al., 2011).

Oils in diatoms have long been thought to contribute to buoyancy of the cells, yet this idea has since been replaced by the hypothesis that ionic pumps drive buoyancy (Ramachandra et al., 2009). Catabolism rates of lipids, such as TAGs, are stable under both light and dark conditions indicating they are not used as immediate food storage for diatoms, rather polysaccharides seem to fill this role (Lancelot & Mathot, 1985). Lipid accumulation usually increases with added stress, such as nitrogen or phosphate starvation, in other microalgae as well as *P. tricornutum* (Hu et al., 2008). This accumulation of lipids, however, often correlates with a decrease in overall biomass (Ramachandra et al., 2009). This would seem to indicate that accumulation of neutral lipids, such as TAGs and hydrocarbons, may instead contribute to the long-term success of an organism under poor growth conditions (Ramachandra et al., 2009).

Even though we have found that *P. tricornutum* does in fact produce short- to medium-chain alkanes and alkenes at higher growth temperature, the ultimate goal is getting the organism to produce them in high enough quantities to be worthwhile for mass production of biofuel. The shorter alkanes that we discovered, octane (C_8H18, *m/z* 114) and undecane ($C_{11}H_{24}$, *m/z* 156), were produced at low relative percentages in the cell at 20°C, only 6% and 5% respectively. The longer alkanes were produced in higher quantities relative to the others, such as nonadecane ($C_{19}H_{40}$, *m/z* 268) at 20°C at an average of 20% and heptadecane ($C_{17}H_{36}$, *m/z* 240) at 30°C at an average of 44%, but may be better suited as diesel replacements because of their chain length. It is known that

diatoms are producers of EPA, DHA, and other polyunsaturated fatty acids, and the emergence of HBIs is now providing more information on the types of neutral lipids produced by members of this extremely large and diverse group of microalgae (Ramachandra et al., 2009). Examination of diatoms to produce very short-chain alkanes (C_2-C_6) is only sparsely studied (McKay et al., 1996), and here we have shown that *P. tricornutum* does possess the ability to produce some short- and medium-chain alkanes, with octane (C_8H_{18}) being the smallest detected, that can be used directly as a fuel substitute. Since nitrogen- and phosphate-limitation are the prevailing means for increasing lipid accumulation, it would be a worthwhile follow-up study to investigate the effects of these growth conditions on the hydrocarbon production of *P. tricornutum* Engineering organisms such as *P. tricornutum* or other microalgae to produce large quantities of these molecules bypasses the need for costly processing, such as hydrocracking or even transesterification, of other types of microalgal oils, such as those produced by *B. braunii*.

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CONCLUSION

Diatoms are one of the largest groups of primary producers in the oceans, yet despite their environmental importance little is known about their plastidial lipid biochemistry. Previously, Yan et al. (2011) found *Skeletonema* species to contain primarily C_{16}/C_{16} and C_{20}/C_{16} forms of mono- and digalactosyldiacylglycerol (MGDG and DGDG, respectively). Likewise, Yongmanitchai and Ward (1993) found *Phaeodactylum tricornutum* to contain primarily C_{16}/C_{16} and C_{20}/C_{20} forms of MGDG and DGDG. In the first study, I sought to relate the results of those previous studies to other diatoms, both in the centrics and pennates, with particular focus on the marennine-producing pennate diatom, *Haslea ostrearia*. To this end, the composition and positional distribution of fatty acids of MGDG and DGDG were examined using positive-ion electrospray/mass spectrometry (ESI/MS). Two centric diatoms, *Skeletonema marinoi* and *Thalassiosira weissflogii*, and the pennate diatom, *P. tricornutum*, contained primarily C_{20}/C_{16} (*sn*-1/*sn*-2) and C_{18}/C_{16} forms of MGDG and DGDG. The other pennate diatoms, *H. ostrearia* and *Navicula perminuta*, contained primarily C_{18}/C_{16} or C_{18}/C_{18} forms of MGDG and DGDG, indicating a previously unrecognized fatty acid diversity in diatom MGDG and DGDG.

The purpose of the second study was to examine the effect of different growth temperatures on the fatty acid compositions of the photosynthetically important galactolipids, MGDG and DGDG, of the "blue" pennate diatom *Haslea ostrearia* and the model pennate diatom, *Phaeodactylum tricornutum*, with the hypothesis that their *sn*-2 fatty acids would be modulated in the same manner as for dinoflagellates. ESI/MS/MS was used to characterize the regiochemistry and relative percentages of the galactolipids of each diatom. At 20°C, *H. ostrearia* and *P. tricornutum* were rich in eicosapentaenoic acid

(EPA; $C_{20:5}$) at the *sn*-1 position and in C_{16} fatty acids at the *sn*-2 position of MGDG and DGDG. At 30°C, however, *H. ostrearia* and *P. tricornutum* contained no EPA or other C_{20} fatty acids, but rather contained higher percentages of C_{18} fatty acids at *sn*-1. At 30°C, no galactolipid in either diatom contained more than 3 unsaturations on any of its fatty acids. While these two species differ in galactolipid composition, they both possess a similar method of acclimating their galactolipids to a higher growth temperature: eliminating the longest and shortest fatty acid chains, as well as decreasing the total number of unsaturations.

The pennate diatom *Phaeodactylum tricornutum* has long been a model species for studies of diatom growth, biochemistry, and lipid accumulation (e.g. triglycerides). Current biofuel prospects face many hurdles to becoming mass-produced, such as ethanol which is lower in energy content and incompatible with the petroleum-based fuel infrastructure in which we live. Consequently, the search for an organism(s) that either produces naturally, or can be engineered and/or manipulated to produce, a fuel source that is both comparable in energy content to petroleum-based fuels and compatible with the current petroleum-based infrastructure is ongoing. In the third study, I have used gas chromatography/mass spectrometry (GC/MS) to examine the hydrocarbons produced by *P. tricornutum* at 20° and 30°C. *P. tricornutum* did indeed produce hydrocarbons similar to those found in petroleum based fuels, namely octane (C_8), undecane (C_{11}), nonadecane (C_{19}), and heneicosane (C_{21}) at 20°C. At 30°C, however, the alkanes produced were instead heptadecane (C_{17}), octadecane (C_{18}), nonadecane (C_{19}), and eicosane (C_{20}). We also observed three alkenes – heptadecene ($C_{17:1}$), octadecene ($C_{18:1}$), and nonadecene ($C_{19:1}$) – which were not present at the lower temperature. If having organisms such as *P.*

tricornutum or other microalgae produce fuel products directly is the goal, then growth temperature may very well be an important factor to consider.

Temperature, therefore, had an effect on the two classes of lipids that were studied, galactolipids and hydrocarbons, in the diatoms that were examined. The potential for projects to branch out from this research are numerous as only a small number of species were examined out of hundreds of thousands of other species of diatoms. As one of the largest, and arguably most important, groups of primary producers on the planet, and certainly in the ocean, it is important to increase our knowledge of the impact potential, and potential applications, of these organisms, whether that be understanding how they can survive constantly changing ocean temperatures or examining how we can manipulate and engineer for the production of beneficial products. Although *P. tricornutum* has previously been used as a model for diatom research due to the abundance of molecular information available, there are thousands of other species that can be researched in the same manner.

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