1 Quantification of chrysolaminarin from the model diatom *Phaeodactylum*

2 *tricornutum*

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10
11 **Abstract**

12 We have established a protocol that allows for parallel quantification of three carbohydrate pools
13 in the marine diatom *Phaeodactylum tricornutum*. This method utilizes a series of extraction and
14 digestion steps followed by the employment of the 3-methyl-2-benzothiazolinone hydrazone
15 (MBTH) reducing sugar assay. Comparing carbohydrate content between hydrolyzed and non-
16 hydrolyzed soluble extracts enables quantification of soluble, nonreducing carbohydrate. The
17 latter fraction contains chrysolaminarin as verified by ¹H-NMR and monomer composition of the
18 purified glucan. We applied this method to investigate carbon partitioning in two experiments.
19 We observed the accumulation of chrysolaminarin during the day and near complete
20 consumption in the dark, supporting its role for fueling heterotrophic metabolism at night. We
21 then observed little change in chrysolaminarin accumulation or consumption during nitrogen
22 starvation, a condition that is known to increase the cellular content of the biofuel precursor
23 triacylglycerol. Overall, this method improves the resolution of major carbohydrate pools in

diatoms, complementing physiological and systems biology studies interested in exploring diatom metabolism.

Keywords

Diatoms, *Phaeodactylum tricornutum*, carbohydrate, chrysolaminarin, nitrogen starvation, carbon partitioning

1. Introduction

Diatoms are important contributors to the biogeochemical cycling of carbon, contributing as much as one-fifth of annual net primary productivity [1]. Diatoms allocate organic carbon into two primary storage metabolites: the neutral lipid triacylglycerol (TAG) and the storage polysaccharide chrysolaminarin [2, 3]. TAG is a major carbon reserve, especially during nutrient stress [4]. TAG productivity is of interest for biofuel production, as these neutral lipids are an important precursor for the production of biodiesel [5]. Diatoms have been identified as a promising platform for biodiesel production because of their relatively high biomass and lipid productivities relative to other algal species [6]. Simultaneously, diatoms synthesize chrysolaminarin as a reserve carbohydrate. Chrysolaminarin has been characterized as soluble polymer consisting of glucose monomers linked by a β -1,3 bond with limited β -1,6 branching [7]. The average molecular weight and number of branches of chrysolaminarin can vary significantly between species [8]. The structure and composition of chrysolaminarin is known; however, the enzymes responsible for its creation and consumption remain broadly uncharacterized [8]. Furthermore, the lack of an established quantification method limits our understanding of this reserve polysaccharide as a fraction of total cellular carbon.

Several studies have addressed the challenge of chrysolaminarin quantification by using a warm-water extraction that quantifies insoluble carbohydrates and soluble carbohydrates in parallel [9, 10]. The diversity of insoluble and soluble carbohydrates produced by diatoms has recently been reviewed [11]. Briefly, insoluble carbohydrates contain structural carbohydrates associated with the frustule such as mannans [12], callose [13], and, in some species, chitin [14]. Soluble carbohydrates contain chrysolaminarin [7], exopolysaccharides [15], and free sugars in metabolism. These insoluble and soluble fractions are typically quantified with total carbohydrate assays such as phenol-sulfuric acid or anthrone methods [16, 17]. These quantification methods do not differentiate between monosaccharides and polysaccharides. Several recent studies have opted to observe diatom carbohydrate biochemistry by measuring total carbohydrates with these assays [18-20]. However, quantifying soluble monosaccharides, soluble polysaccharides (chrysolaminarin), and insoluble polysaccharides in parallel with a reducing sugar assay [21] will provide more insight into carbon partitioning following genetic manipulation or physiological conditions.

Metabolic engineering strategies in many photoautotrophs seek to increase the flux of carbon and energy towards molecules of interest [5, 22]. The proportions of carbon found in the two major carbon reserves of algae, neutral lipids and storage polysaccharides, can be manipulated through changing physiological status or by disruption of biosynthetic pathways. Nitrogen starvation is one of the best-characterized examples of manipulating metabolism: increased TAG accumulation has been observed in a broad range of organisms from diatoms [23-27] to green algae [27-30] when nitrogen is removed from the medium.

Carbon partitioning also can be altered by genetic engineering. It is known that some proportion of cellular organic carbon is allocated as carbon reserves (both TAG and storage

polysaccharides). It was reasoned that disrupting polysaccharide metabolism might further increase the accumulation of TAG, as the displaced storage carbon from polysaccharides had to be partitioned somewhere in metabolism. For instance, mixotrophically grown starchless *Chlamydomonas* mutants accumulated even more TAG than wild type during nitrogen starvation [31, 32]. Therefore, it is rational to suggest that manipulating chrysolaminarin metabolism may enhance TAG productivity in diatoms. Several groups have investigated this possibility in diatoms with a reverse genetics approach by knocking down or knocking out putative enzymes in chrysolaminarin biosynthesis [33, 34], or by blocking β -1,3 glucanase activity [35]. All of these studies reported an increase in TAG content; however, they could only infer a reduction in chrysolaminarin accumulation. Conclusions about carbon repartitioning from chrysolaminarin to other carbon sinks have broadly been limited by the inability to specifically quantify chrysolaminarin from other major carbohydrate pools.

Our primary objective with this study was to establish a method to quantify chrysolaminarin and thereby improve the biochemical toolkit of diatoms to study central carbon metabolism. The method described here is based off of the sensitive and accurate 3-methyl-2-benzothiazolinone hydrazone (MBTH) assay [21, 36]. The MBTH assay has already been applied to a range of carbohydrate quantification applications including field samples [37], glycolytic assays [38, 39], and total carbohydrate content of algae [40]. It provides considerably greater accuracy and precision compared to the commonly used anthrone and phenol-sulfuric acid carbohydrate assays [40]. This assay is coupled to a workflow that quantifies three major carbohydrate pools from *Phaeodactylum tricornutum*, including a soluble, nonreducing fraction that contains chrysolaminarin. We then applied this workflow to quantify the changes in carbon partitioning to storage metabolites during nitrate starvation on a light/dark cycle. Our data

indicate that, during nitrate starvation, chrysolaminarin per cell decreases and that TAG represents the dominant reserve carbon molecule.

2. Materials and Methods

2.1. Extraction and analytical chemistry of carbohydrates

2.1.1. *Culturing conditions*

Batch cultures of axenic *Phaeodactylum tricornutum* CCAP 1055/1 (hereafter, *Phaeodactylum*) were grown in 1 L Roux flasks with 0.8 L of artificial seawater (Instant Ocean, 35 ‰ salinity). Nutrients were added per the stoichiometry described by Guillard [41], but at a 2.3-fold higher concentration to mitigate any nutrient limitation. Silicon was omitted from the medium, as it is not required for *Phaeodactylum* growth. Light was provided in a 12:12 day/night cycle, where daytime irradiance was set at $420 \pm 20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (LI-COR light meter, LI-250A, 2 π sensor). Five replicate cultures were grown at 18 °C and were mixed by sparging with 1 L min⁻¹ air. Cultures were maintained in exponential growth for at least two days prior to the start of the experiment with serial dilutions.

2.1.2. *Extraction and purification of soluble carbohydrate*

The 4 L volume was harvested at dusk during exponential growth and cells were pelleted by centrifugation ($3220 \times g$, 10 min, 18 °C) in 50 mL conical tubes. All harvested cells were combined into a single pellet, resuspended with 10 mL deionized water, and incubated at 50 °C for 30 min. The suspension was spun ($3220 \times g$, 10 min, 18 °C) and the 10 mL warm water extract was transferred to a fresh 50 mL conical tube. Then, 40 mL of 95 % ethanol was added to the 10 mL warm water extract, inverted, and allowed to form a precipitate overnight at -20 °C [7]. Soluble, total carbohydrate content was monitored throughout the purification procedure

116 (described below, 2.2.3. – 2.2.4.). The precipitate was pelleted by centrifugation ($3220 \times g$, 10
117 min, 4 °C), supernatant discarded, and the pellet was thoroughly resuspended in 10 mL 95%
118 ethanol by vigorous pipetting. This process was repeated once more to wash the pellet with 95 %
119 ethanol twice in total. After the second ethanol wash, the pellet was resolubilized with 5 mL
120 deionized water. The resolubilized precipitate was enzymatically treated: a simultaneous DNase
121 (Thermo Scientific, #EN0521) and RNase (Thermo Scientific, #EN0201) digest was performed
122 for 1 h at 4 °C, followed by a $90 \mu\text{g mL}^{-1}$ Proteinase K treatment (Fisher Scientific, BP1700-100)
123 for 2 h at 4 °C. Then, 4 volumes of 95 % ethanol were added to this aqueous solution and
124 allowed to precipitate overnight at -20 °C. Two 95% ethanol washes were performed the
125 following day, and the pellet was resuspended with deionized water to a final volume of 3 mL.
126 This 3 mL solution transferred into a 2K MWCO Slide-A-Lyzer dialysis cassette (Thermo
127 Scientific, #87718) and was dialyzed against deionized water for 24 h at 18 °C with continuous
128 stir bar mixing. The dialysis volume was $3 \times 0.6 \text{ L}$, where deionized water was replaced at 2 h
129 and 4 h. The dialyzed retentate was passed through a 5 mL DEAE FF column (GE Healthcare,
130 #17-515-01) on an ÄKTA start FPLC (GE Healthcare). Protein content was monitored during
131 elution using an A280 detector on the FPLC. A 0 – 1 M NaCl gradient was applied using a 1 M
132 NaCl buffer and 5 mL fractions were collected. Carbohydrate-containing fractions were pooled,
133 frozen in liquid nitrogen, and lyophilized overnight. A polishing step took place by resuspending
134 the lyophilized powder with deionized water to a final volume of 0.5 mL and applied to a
135 Superdex 75 10/300 GL (GE Healthcare, #17-5174-01). One mL fractions were eluted using a
136 flow rate of 0.5 mL min^{-1} and carbohydrate enriched fractions pooled, frozen, and lyophilized as
137 described above, which yielded a purified polysaccharide sample used for compositional and

structural analyses. Laminarin from *Laminaria digitata* was purchased from Sigma (#L-9634) and was directly used as a control for compositional and structural analyses.

2.1.3. Compositional analysis with alditol acetate derivatization and GC/MS

Compositional analysis of purified carbohydrate samples was performed using an established GC/MS approach [42]. Briefly, 1.0 µg of the internal standard, 3-*O*-methylglucose, was added to each sample: laminarin, the purified soluble glucan from *Phaeodactylum*, and the neutral sugar standard (rhamnose, arabinose, ribose, fucose, myo-inositol, mannose, galactose and glucose; 5 µg each). Dried samples were hydrolyzed with 250 µL 2 M trifluoroacetic acid (TFA) for 2 h at 120 °C. After cooling to room temperature (RT), samples were dried under nitrogen gas (N₂). The dried, hydrolyzed samples were reduced with 200 µL 10 mg mL⁻¹ sodium borodeuteride in 1:1 of 1 M ammonium hydroxide and 95 % ethanol for 2 h at RT, which was then terminated by adding 4 - 5 drops of glacial acetic acid. After drying the reduced samples under N₂, 200 µL 10% glacial acetic acid in methanol were added, and then were dried under N₂. This process was repeated 4 - 5 times. To the completely dried samples, 100 µL acetic anhydride was added, incubated at 100 °C for 1 h, and then were gently dried under N₂. Two mL chloroform and 1 mL deionized water (v/v, 2:1) were added to the acetylated samples to extract the derivatized products by centrifugation (1000 × g, 1 min, RT). After removing the upper aqueous layers, 1 mL deionized water was added to the lower organic phase to repeat the extraction 4 more times. The organic phase was gently dried under N₂ and resuspended in 50 µL chloroform for gas chromatography/mass spectrometry (GC/MS) analysis. GC/MS analyses were carried out using a CP 3800 gas chromatograph (Varian) equipped with an MS320 mass spectrometer. Helium was used as the carrier gas with a flow rate of 1 mL min⁻¹. The samples were run on a DB 5 column (30 m × 0.20 mm i.d.). The oven temperature was held at 50 °C for

1 min and programmed at 30 °C min⁻¹ to 150 °C and then programmed at 5 °C min⁻¹ to 275 °C.

Chromatographs were exported to Sigmaplot for analysis.

2.1.4. Structural analysis and interpretation with ¹H-NMR

Proton NMR spectroscopy at 500 MHz of the glucan dissolved in DMSO-d₆ was done at 80 °C with a Varian-Inova NMR Spectrometer by using a switchable, 5mm broadband probe. The spectral window was 8kHz and the acquisition time was 2.0 seconds. 32 signal averaging transients were applied with a 22.5 degree tip angle in a simple one-pulse-acquire sequence. There was a relaxation delay of 10 seconds between transients. The free induction decay, FID, was Fourier transformed and the resulting spectral data resolution was 0.5 Hz per point. The DMSO-d₅H₁ impurity was used as an internal spectral reference set to 2.5ppm. Peaks resolved in the spectra were annotated based on their chemical shift and the area under the annotated peaks was determined to infer structural properties of the β-1,3 glucan as previously described [43].

2.2. Diel light regime experiment

2.2.1. Growth conditions, medium, and cell harvesting

Phaeodactylum was grown in medium as described in 2.1.1 but instead maintained in 125 mL Erlenmeyer flasks. Triplicate cultures were grown at 18 °C and were constantly mixed on a shaking platform at 120 rpm. Light was provided in a 16:8 day/night cycle, where daytime irradiance was maintained at 325 ± 15 μmol photons m⁻² s⁻¹ (LI-COR light meter, LI-250A, 2 π sensor). *Phaeodactylum* cells (~1.0 x 10⁸) were harvested by centrifugation (3220 × g, 10 min, 18 °C) in 50 mL conical tubes. The supernatant was discarded and the pellet was stored at -80 °C until warm water extraction.

2.2.2. Warm water extraction of soluble carbohydrates

We followed the warm water extraction described by Chiovitti et al. [10], but at a higher incubation temperature. Frozen cell pellets were thawed on the day of analysis, suspended in 1.5 mL deionized water (18.2 mΩ), placed in microcentrifuge tubes, and incubated at 50 °C for 15 min to extract soluble carbohydrates. Insoluble carbohydrates and soluble carbohydrates were separated by centrifugation (20,000 × g, 5 min, RT), and the 1.5 mL soluble fraction was transferred to a new microcentrifuge tube.

2.2.3. Sulfuric acid hydrolysis of carbohydrate fractions

Sulfuric acid hydrolysis and quantification of carbohydrates were performed as described by Van Wychen and Laurens [40], with several changes to the protocol to enable quantification of multiple carbohydrate fractions. Immediately after the warm water extraction described above, the insoluble pellet was resuspended with 1 mL deionized water and transferred to a labeled, glass microwave vial (VWR, #89079-404). A 1 mL aliquot of the soluble fraction was also transferred to a labeled, glass microwave vial. The remaining 0.5 mL soluble carbohydrate sample was reserved at 4 °C until quantification. 250 μL sulfuric acid (72% (w/w); Fluka, #00647) was added to each sample, capped (Wheaton, #224100-203), vortexed, and incubated at RT for 1 h. After the incubation, the tubes were uncapped and 6 mL deionized water was added to each sample, bringing the final volume to 7.25 mL. The vials were recapped, crimp-sealed with 20 mm aluminum caps (Wheaton, #224193-01), and placed in an autoclavable rack in a pressure cooker for 1 h (121 °C, 83 kPa). After the pressure equalized, vials were removed from the pressure cooker and cooled to RT. A 2 mL aliquot was taken from each acidified sample and placed in a labeled 50 mL conical tube and was neutralized (pH 6 – pH 8) with calcium carbonate (Sigma-Aldrich, #C6763), verified by placing a few drops of solution on pH indicator strips (MColorpHast, EMD Millipore, #109535). Neutralized solution was then filtered through a

nylon filter (0.2 μm pore size; Fisher, #09-719-006) to remove precipitates. Neutralized, filtered solutions were stored at 4 °C and used for quantification within 24 h.

2.2.4. *Quantification of carbohydrate fractions*

Quantification of carbohydrates using the MBTH method was performed as described previously [40], with some modifications outlined below. A 250 μL sample was added to a glass vial (Pyrex, #9820-10), to which 250 μL 0.5 M NaOH and 500 μL of a freshly prepared 1:1 (v/v) solution of 3 mg mL^{-1} MBTH:1 mg mL^{-1} dithiothreitol (Sigma-Aldrich, #129739; Sigma-Aldrich #D0632) were added, in that order. Glass vials were capped, vortexed, and incubated at 80 °C for 15 min. 500 μL ferric solution (0.5 % (w/v) ammonium iron (III) sulfate (Acros Organics, #205880500), 0.5 % (w/v) sulfamic acid (Sigma-Aldrich, #242772) in 0.25 M HCl) was added to each sample on the heat block. Samples were vortexed, removed from the heat block and allowed to cool for 15 min at RT. These samples were diluted with 1250 μL deionized water, and 1 mL was transferred to 1 cm pathlength acrylic cuvettes to determine absorbance at 620 nm. Carbohydrate concentrations were determined from a standard curve of glucose concentrations ranging from (0 to 0.050) mg mL^{-1} prepared in parallel with each set of experimental samples.

The soluble fraction that was hydrolyzed, neutralized, and filtered (see 2.3.4.) generated a value for total soluble carbohydrates. Similarly, the insoluble fraction that was hydrolyzed, neutralized, and filtered generated a value for total insoluble carbohydrates. The soluble fraction that was reserved after warm water extraction (see 2.3.3.) generated a value for soluble, reducing carbohydrates only. Subtracting the concentration of soluble, reducing carbohydrates from soluble, total carbohydrates yielded soluble, nonreducing carbohydrates. Dividing these concentrations by the total number of cells harvested for the respective experimental sample

yields carbohydrate fractions on a per cell basis. All carbohydrate fractions are reported as average pg glucose equivalents (g.e.) per cell.

2.3. Nitrate depletion experiment

2.3.1. *Growth conditions and media*

Phaeodactylum was grown in 1 L Roux flasks in 0.8 L of artificial seawater (Instant Ocean, 35 ‰ salinity). Nutrients were added as described by Guillard [41], except for the nitrate deplete media where nitrate was omitted. Silica was omitted from the media. Light was provided in a 12:12 day/night cycle, where daytime irradiance was set at $420 \pm 20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (LI-COR light meter, LI-250A, 2π sensor). Five replicate cultures were grown at 18 °C and were mixed by sparging with 1 L min⁻¹ air. Cultures were maintained in exponential growth for at least two days prior to the start of the experiment with serial dilutions. The experiment was carried out for 4 days. Harvests extracted appropriate volumes (described below) to quantify: carbohydrates, total organic carbon and total nitrogen, chlorophyll, and triacylglycerol. Nitrate replete cultures were harvested at dusk and dawn at 0 and 0.5 days. Culture media was exchanged at 0.6 days by spinning down 400 mL of culture ($3220 \times g$, 10 min, 18 °C), washing pellets twice with filtered artificial seawater, and resuspending the algae pellets in 400 mL nitrate deplete media. Nitrate deplete cultures were harvested at dusk and dawn at 2.5, 3.0, 3.5, and 4.0 days. Flow cytometry and chlorophyll *a* fluorescence measurements were taken every 12 h, corresponding to dusk and dawn during the entire nitrate depletion experiment.

2.3.2. *Flow cytometry: cell counts, chlorophyll autofluorescence and forward scattering*

We used an Accuri C6 flow cytometer (BD) to determine cell density, chlorophyll autofluorescence per cell, and forward scattering per cell. 2 mL of culture were passed through a

30 μm filter (Myltenyi Biotec, #130-101-812) to remove any debris prior to analysis and diluted in filtered artificial seawater to maintain less than 2000 detected events sec^{-1} . The flow rate was 35 $\mu\text{L min}^{-1}$, and the core size was 16 μm . Relative chlorophyll autofluorescence was determined by excitation with a 488 nm laser (emission > 670 nm). *Phaeodactylum* cell counts μL^{-1} were determined by gating for particles with chlorophyll autofluorescence.

2.3.3. Nitrate depletion experiment cell harvesting

Cells harvested during nitrate depletion did not adequately pellet in 50 mL conical tubes; therefore, we collected cells by filtration. About 2.0×10^8 *Phaeodactylum* cells were harvested by filtering a culture volume (ranging from 50 to 150 mL, dependent on cell density) onto a polycarbonate filter (EMD Millipore, #ATTP02500). Filters were then placed in a beaker and cells were removed from the filter by repeated pipetting with 2 mL filtered artificial seawater. The 2 mL suspension was transferred into two 1 mL aliquots in microcentrifuge tubes. These resuspended cells were pelleted by centrifugation ($20,000 \times g$, 10 min, 18 °C) and the supernatant was meticulously removed. These pellets were stored at -80 °C until further processing. One pellet was used for quantification of carbohydrate fractions (described above) and the other was used for triacylglycerol quantification (described below).

2.3.4. Total organic carbon and total nitrogen quantification

Total organic carbon (TOC) and total nitrogen (TN) were determined using a Shimadzu TOC-L Laboratory TOC Analyzer. TOC was determined by the difference method, where inorganic carbon (IC) was subtracted from total carbon (TC) to yield TOC. Ten mL of culture was harvested at dusk and dawn during the nitrate depletion experiment and placed into 50 mL conical tubes. These samples were pelleted by centrifugation ($12000 \times g$, 15 min, 18 °C) and washed once with filtered artificial seawater, then resuspended in 10 mL deionized water. A 1

mL aliquot was taken to determine cell density by flow cytometry. The remaining 9 mL was frozen at -80 °C until the day of analysis. All glass and plasticware was acid-washed with 10 % HCl prior to use to remove any residual organic carbon. On the day of analysis, the 9 mL samples were thawed to room temperature. Eight mL was transferred into TOC glass vials and were diluted to a final volume of 40 mL with deionized water. The injection volume was 100 µL, and the instrument performed technical replicates of the injection such that duplicate values of less than 10% difference were used to determine TC, IC and TN for each sample. TC, IC, and TN concentrations were then divided by the number of cells in the 8 mL sample to determine TOC and TN per cell.

2.3.5. Chlorophyll quantification

10 mL of *Phaeodactylum* cells were collected and stored at -80 °C. Samples were thawed the day of analysis and filtered through a glass fiber filter (EMD Millipore, AP2001300). Pigments were extracted from glass filters by resuspension with 1 mL methanol, vortexed for 15 s, and incubated in the dark for 15 min. Cell debris and residual glass fibers were removed by centrifugation ($15000 \times g$, 10 min, 4 °C) and the pigment-containing supernatants were pipetted into disposable acrylic cuvettes for analysis. No visible color remained on the filters. Chlorophyll *a* and chlorophyll $c_1 + c_2$ were determined using established spectrophotometric methods [44]. Chlorophyll *a* per cell was determined by dividing the chlorophyll *a* concentration by the total number of cells in the harvested sample, calculated from cell density and the volume extracted.

2.3.6. Monitoring neutral lipids with BODIPY and triacylglycerol quantification

Neutral lipid accumulation patterns were observed during the nitration depletion experiment using the fluorescent dye BODIPY (Ex = 503 nm, Em = 512 nm, Life Technologies, #D-2184). A single solution of 100 µg mL⁻¹ BODIPY in dimethyl sulfoxide was prepared and

divided into single-use aliquots stored at -80°C for all timepoints during the nitrate depletion experiment to minimize variability of the dye from handling and freeze-thaw cycles. Samples were prepared for flow cytometry as described above. A BODIPY aliquot was thawed immediately before analysis and $6.7\text{ }\mu\text{L}$ of the BODIPY solution was added to a 1 mL filtered culture sample, yielding a final concentration of $0.067\text{ }\mu\text{g mL}^{-1}$ [45]. Samples were vortexed for 3 s , incubated in the dark for 4 min , then briefly vortexed again prior to analysis by flow cytometry. BODIPY fluorescence was determined by excitation with a 488 nm laser and collected by a $533/30\text{ nm}$ bandpass filter, enabling quantification of mean BODIPY fluorescence per cell.

Triacylglycerols (TAG) were quantified from a neutral lipid extraction, as described previously [46]. Briefly, cell pellets were prepared for analysis (see 2.3.2) and neutral lipids from these pellets were extracted via solid phase extraction, as described previously [47]. Eluted TAG samples were placed in a random order on a silica plate for thin layer chromatography and quantification. TAG was quantified from a standard curve of tripalmitin (1,2,3-trihexadecanoyl-glycerol, TAG 16:0 16:0 16:0, Avanti Polar Lipids, #111000) using ImageJ, version 1.48 (<http://imageJ.nih.gov/ij/>). Values reported as tripalmitin equivalents (t.e.) per cell.

2.3.7. *Chlorophyll a fluorescence measurements*

Five mL of cell culture was harvested at dawn and dusk timepoints during the nitrate depletion experiment for variable chlorophyll fluorescence measurements to determine F_v/F_m with a Walz DUAL-PAM 100 fluorometer. Samples were acclimated at $9\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$ for 30 min to relax nonphotochemical quenching prior to F_v/F_m measurement [48]. Samples were filtered onto a glass fiber filter (EMD Millipore, AP2001300) for measurement. Measuring light

intensities were adjusted so that the baseline fluorescence was approximately 0.20 V prior to the saturating light pulse.

2.4. Statistical analysis

A paired student's t-test ($\alpha = 0.05$) was used to compare quantities of carbohydrate pools between dawn and dusk. For the nitrate depletion timecourse, a repeated measures one-way ANOVA ($\alpha = 0.05$) followed by a Tukey's HSD post-hoc test was performed to describe statistically different groups. All statistical analysis was performed using Sigmaplot (Systat Software).

3. **Results**

3.1. Analytical chemistry of soluble polysaccharide extracted from *Phaeodactylum*

Three major carbohydrate fractions were resolved with our method: insoluble; soluble, reducing; and soluble, total carbohydrates (Figure 1). A critical methodological assumption is that chrysolaminarin is present in the soluble, nonreducing carbohydrate determined from subtracting soluble, reducing sugars from soluble, total carbohydrates. To test this assumption, we set out to analyze the soluble, nonreducing carbohydrate. We tested a range of warm water temperatures in order to extract soluble carbohydrates from *Phaeodactylum*. We found that the greatest values of soluble carbohydrate were found at extraction temperatures above 50 °C, which therefore represents an appropriate soluble carbohydrate extraction temperature for *Phaeodactylum* (Supplemental Figure 1).

We enriched a *Phaeodactylum* polysaccharide from this soluble extract for compositional and structural analysis. The first round of precipitation contained 72 mg glucose equivalents (g.e.) carbohydrate from the combined 4 L batch culture as determined by MBTH quantification

of soluble, total carbohydrate. 57 mg g.e. of soluble, total carbohydrate remained after enzymatic digests, ethanol precipitation, and dialysis, and 45 mg g.e. remained after two rounds of FPLC purification (Supplemental Figure 2). FPLC fractions were pooled, dialyzed and lyophilized, yielding a dry weight of 42.1 mg of material that was 97.6 % pure carbohydrate by dry weight. The final yield of purified polysaccharide was 58 % of the initial soluble extract quantity, with most of the losses occurring during the early ethanol precipitation steps prior to FPLC purification.

Monomer analysis of the purified polysaccharide was determined via GC/MS of alditol acetate sugar derivatives (Figure 2A). Our positive control, laminarin, was comprised of glucose (Figure 2B). The *Phaeodactylum* purified polysaccharide was composed of glucose (Figure 2C). The internal standard, 3-O-methylglucose, was observed in all samples.

We also performed structural analysis of the purified polysaccharide using ^1H -NMR. This analysis relied on existing annotations and chemical shift assignments [43]. A model β -glucan with individually labeled anhydrous glucose units (AGU) is shown in Figure 3A for a structural reference. Protons from each of these unique anhydrous glucose units are resolved in the β -glucan ^1H -NMR spectra as a doublet at an expected chemical shift. The spectra of laminarin (Figure 3B) and *Phaeodactylum* purified polysaccharide (Figure 3C) are presented. The largest annotated doublet corresponded to the backbone chain (BC) at ~4.54 ppm in both spectra. A doublet for the terminal side chain AGU is not observed in the *Phaeodactylum* soluble polysaccharide, indicating a single AGU for the side chain. Analysis of the integrals of the doublets permits structural inference of the glucans (Table 1). Laminarin has 28 glucose residues as determined by degree of polymerization (DP) with a degree of branching (DB) of 0.063. These values are higher than the *Phaeodactylum* purified polysaccharide with 17 glucose

residues and a DB of 0.015. The number of branches per each molecule can be estimated by multiplying DP and DB. Laminarin has about 2 branches per polysaccharide while only about one in four of the *Phaeodactylum* purified polysaccharide has a side chain.

3.2. Accumulation of carbohydrate fractions differs in day/night cycles

We compared our three experimentally resolved carbohydrate fractions at dawn and dusk (Figure 4). There was no significant change of insoluble carbohydrates per cell between dawn and dusk ($p = 0.80$). A significant decrease of soluble, reducing carbohydrates was observed between dawn and dusk ($p < 0.05$). However, the largest relative and absolute differences observed were for soluble, nonreducing carbohydrates. This component dropped from 1.56 ± 0.25 pg g.e. per cell at dusk to 0.06 ± 0.03 pg g.e. per cell at dawn ($n = 3$, $p < 0.05$).

3.3. Nitrate depletion cell physiology and carbon partitioning into carbohydrate fractions

We investigated cell physiology during the first 3 days of nitrate depletion. Cell densities increased in nitrate replete medium, but the increase of cell densities stopped 2 days following transfer into nitrate deplete medium (Figure 5A). The forward scatter (FSC) measured by flow cytometry is a proxy for cell size, since the relationship between FSC and cell size is not linearly proportional for pennate diatoms [49]. The FSC of *Phaeodactylum* oscillated in nitrate replete medium, with a maximum at dusk and a minimum at dawn (Figure 5B). The oscillation pattern ceased after 2 days in nitrate deplete medium.

TOC per cell significantly decreased in the dark under nitrate replete conditions but the daily increase in TOC was not observed in nitrate deplete conditions (Figure 5C, $p < 0.05$). The decrease of TN per cell in nitrogen replete medium at night was small but statistically significant

(Figure 5D, $p < 0.05$). TN per cell decreased during nitrogen deprivation to about 0.44 pg TN per cell ($p < 0.05$).

F_v/F_m , a measurement of the quantum efficiency of photosystem II [50], dropped from 0.6 to 0.2 after two days of nitrate deprivation (Figure 5E, $p < 0.05$). Chlorophyll *a* was maintained between 0.13 – 0.14 pg per cell in nitrate replete media but decreased after 2 – 3 days without nitrate (Figure 5F, $p < 0.05$). No significant difference was observed for the chlorophyll $c_1 + c_2$: chlorophyll *a* ratio at any timepoint during the experiment (Figure 5G, $p = 0.19$).

We observed the partitioning of carbon within different carbohydrate fractions and neutral lipids during nitrate depletion. Soluble, nonreducing carbohydrate dropped significantly in the dark from 2.32 pg g.e. per cell to 0.36 pg g.e. per cell but remained between 1.04 and 1.34 pg g.e. per cell 2 – 3 days after nitrate removal (Figure 6A, $p < 0.05$). Soluble, reducing sugars also significantly decreased at night in nitrate replete medium (Figure 6B, $p < 0.05$). Soluble, reducing sugars accumulated at slightly lower quantities after 2 – 3 days in nitrate deplete medium than the maximum in nitrate replete medium. A small, significant drop of insoluble carbohydrate was observed in nitrogen replete growth between dawn and dusk, but also remained at a steady level in nitrate deplete conditions, between 0.82 and 0.86 pg g.e. per cell (Figure 6C, $p < 0.05$). The amount of soluble, reducing sugars did not change throughout nitrogen starvation (Figure 6D, $p < 0.05$).

BODIPY fluorescence per cell, which is commonly used as an indicator of neutral lipid content, oscillated with maxima at dusk and minima at dawn for the entire experiment (Figure 6E, $p < 0.05$). However, quantification of TAG per cell suggested that the oscillating neutral lipid abundance is limited to nitrate replete conditions (Figure 6F, $p < 0.05$). During nitrate starvation, TAG per cell accumulated to about 6 pg t.e. per cell and did not differ between dawn and dusk.

Soluble, nonreducing sugars and TAG were normalized for their mass-fraction of carbon, 0.4 units (glucose, $C_6H_{12}O_6$) and 0.76 units (tripalmitin, $C_{51}H_{98}O_6$) respectively (Figure 7). Carbon partitioning into soluble, nonreducing sugar was comparable to triacylglycerols at dusk under nitrate replete conditions, accounting for 6.9 % and 10 %, respectively. However, after 2 – 3 days of nitrate removal, soluble, nonreducing carbohydrate only represents about 5.5 % of TOC whereas TAG dominates carbon partitioning, accounting for about 45 % of TOC.

4. Discussion

4.1 Soluble, nonreducing carbohydrate fraction contains chrysolaminarin

Chrysolaminarin was originally identified as a soluble polysaccharide, entirely composed of glucose, with limited branching [7]. Our extraction workflow began by extracting saccharides from whole cells at 50 °C. This bulk extract does not contain any signature components of *Phaeodactylum* extracellular polysaccharides, which are composed of mannose and glucose with complex linkages and branching [51]. Instead, our isolated glucan from the soluble extract was entirely composed of glucose (Figure 3), agreeing with past studies of chrysolaminarin [7, 52]. The purified glucan exhibited 1H -NMR signatures characteristic of β -1,3 glucans (Figure 4). However, purification accounted for a loss of 15 mg g.e of. uncharacterized glucan from dialyzed retentate to purified glucan, or about 26 %. It is possible that this lost fraction contains some alternative soluble glucose polymer whose identity is unknown.

The 1H -NMR spectrum also permits inference of structural characteristics of the glucan. The integrated, annotated peaks can be used to determine the degree of polymerization (DP) and

the degree of branching (DB) [43]. Structural features significantly vary by diatom species, even between closely related organisms [11, 51-53]. We found that *Phaeodactylum*'s chrysolaminarin is smaller and lightly branched compared to laminarin, which is consistent with earlier observations. [7] (Table 1). The structure of the β -1,3/ β -1,6 glucan means that for an average molecule of chrysolaminarin, 16 of 17 glucose units are non-reducing sugars (94 %) (Table 1). Compositional and structural analysis of the *Phaeodactylum* glucan supports our assumption that the soluble, nonreducing carbohydrate fraction contains chrysolaminarin. We recommend similar analytical steps be taken when applying this method to species other than *Phaeodactylum*.

Chrysolaminarin, is thought to serve as a respiratory substrate in the dark [46]. Our results demonstrate that soluble, nonreducing carbohydrate dramatically decreases between dusk and dawn, especially relative to the other carbohydrate pools (Figure 4). We also found this fraction significantly increased with growth irradiance (Supplemental Figure 3). These observations further support our assertion that the soluble, nonreducing fraction is mostly chrysolaminarin. We note that our values are lower than those found by Chauton et al. [18] from *Phaeodactylum* cells entrained to day/night cycles. The extraction methods used by Chauton et al. [18], which contains dilute sulfuric acid, have been shown to extract other non-glucose containing carbohydrates in addition to chrysolaminarin [53] and the colorimetric assay utilized to quantify reducing sugars (phenol-sulfuric acid) provides poor precision for a complex mixture of sugars [55]. In light of our analytical chemistry and physiology results for the soluble, nonreducing carbohydrate, we suggest that this fraction represents a good proxy for quantifying chrysolaminarin and hereafter refer to this as chrysolaminarin.

4.2 Repartitioning carbohydrate pools during nitrogen starvation.

We sought to understand the dynamics of chrysolaminarin storage in response to nitrogen starvation. Our biochemistry and photophysiology data show a daily oscillation in *Phaeodactylum* cell composition and size prior to exchange into nitrogen free media. Cell density increased (Figure 5A) and cell size (assumed to be proportional to forward scatter) oscillated depending on time of day (Figure 5B). The quantum efficiency of photosystem II remained high (Figure 5E), as did chlorophyll *a* per cell (Figure 5F). We, and others, have previously observed synchronized cell division in *Phaeodactylum* growing in a day/night cycle [18, 46]. An increase of cell TOC during the day, particularly as chrysolaminarin and TAG, (Figure 6) provides carbon skeletons that support cell division and heterotrophic metabolism at night during nutrient replete growth. The consumption of these substrates in the dark to fuel heterotrophic metabolism has been described previously [46, 56].

The daily oscillation observed in nutrient replete conditions ceased in nitrogen-deplete conditions. This was observed after 2 – 4 days in nitrate-free medium. Increases in cell concentration slowed (Figure 5A) and oscillations in cell size stopped (Figure 5B). While TN (Figure 5D), F_v/F_m (Figure 5E), and chlorophyll *a* (Figure 5F) dramatically decreased, the chlorophyll $c_1 + c_2$: chlorophyll *a* ratio did not change (Figure 5G), in agreement with previous nitrate starvation observations [57].

The patterns of carbon partitioning between carbohydrate fractions and TAG during nitrogen starvation were unforeseen. The dusk/dawn cellular content of carbohydrate did not change during nitrogen starvation and did not increase relative to nutrient replete growth (Figure 6). This was also observed for each experimentally resolved carbohydrate pool, including the cellular content of chrysolaminarin (Figure 6B, timepoints 4-6) which only represented 5.5 % of total cellular carbon during nitrogen limitation (Figure 7). TAG content reached its highest

values on a per cell basis (Figure 6F) and also as a total fraction of the TOC (Figure 7). These data show that TAG, not chrysolaminarin, is the primary repository of storage carbon for *Phaeodactylum* during nitrate starvation in day:night cycles.

While it has been determined that total carbohydrate in *Phaeodactylum* decreases during nitrate starvation [19, 56], the dynamics of individual carbohydrate pools have previously only been inferred through proteomics and transcriptomics. For instance, Hockin et al. [24] performed a comparative proteomics experiment at the onset of nitrogen starvation, where they observed a variety of enzymes predicted to participate in carbohydrate metabolism significantly changed. Some of the proteins that increased in relative abundance were glycolysis-specific, suggesting a net shift of carbon from chrysolaminarin reserves into free sugars for metabolism. Transcripts for glycolytic enzymes also increase in abundance during nitrate depletion [19, 26, 35]. Mus et al. [59] also noted an increased abundance of transcripts that are bioinformatically predicted to be related to chrysolaminarin metabolism during nitrate starvation. We note that all experimentally resolved carbohydrate fractions decreased during N-starvation in our study so we posit that some of these transcripts might be associated with the metabolism of insoluble carbohydrates.

One proposed strategy to enhance lipid productivity in algae is to interfere with polysaccharide synthesis [5]. Indeed, photoheterotrophically grown *Chlamydomonas* strains that are unable to produce starch greatly increase the amount of cellular carbon in TAG [31, 32]. This change in carbon partitioning may be further enhanced by nitrogen deprivation, when TAG is significantly accumulated [4]. We found that *Phaeodactylum* only accumulates 4.9 – 6.0 % of cellular carbon as chrysolaminarin during nitrate starvation, a relatively small fraction compared to the TAG accumulation of 43 – 50 % of cellular organic carbon (Figure 7). We note that a large proportion of cellular carbon is used in protein and structural lipids [46], but we did not quantify

those pools in this study. Therefore, the rational engineering strategy of manipulating storage carbohydrate metabolism to increase TAG productivity in *Phaeodactylum* might not be appropriate. However, it is important to note that the accumulation of soluble carbohydrate varies significantly by algal species [60]. For instance, *Skeletonema costatum* accumulated soluble carbohydrate during nitrate depletion in a diel light regime [61]. Additionally, N-starved *Isochysis sp.* accumulated total carbohydrate without daily oscillations compared to N-replete cultures [25], but it is unclear how much of the observed carbohydrate was chrysolaminarin in either of these studies. We caution that careful attention to species-specific physiology and growth conditions must be made when predicting the potential yield changes associated with the engineering of metabolism.

5. Conclusions

We report a method that enables parallel characterization of insoluble; soluble, reducing; and soluble, nonreducing carbohydrates, representing an improvement over existing total carbohydrate assays. We have established that the soluble, nonreducing carbohydrate resolved by our MBTH-based method contains chrysolaminarin. Quantification of chrysolaminarin in parallel with other carbohydrate pools is a valuable addition to the biochemical toolkit available for investigating diatom metabolism. We found that high accumulation of chrysolaminarin in *Phaeodactylum* requires nutrient replete conditions, high irradiance, and occurs at dusk in a diel light regime. We illustrated the utility of this method by showing that: 1) chrysolaminarin accumulates at the end of the day and is consumed by dawn and 2) chrysolaminarin is not preferentially accumulated during nitrogen limitation. So, we stress that estimates of carbon partitioning to biofuel precursors should be made on cultures grown in day/night cycles. We also

526 suggest that rerouting of carbon flux away from chrysolaminarin reserves is not the most prudent
527 biological engineering strategy for maximizing TAG productivity in this organism.

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529

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720

721 **Table 1: Structural analysis of laminarin and soluble**
722 **polysaccharide extracted from *P. tricornutum* using**
723 **annotated β -1,3 glucan signatures.**

	Kim et al. (2000)	This study	
<i>substrate</i>	<i>laminarin</i>	<i>laminarin</i>	<i>soluble glucan (P.t.)</i>
DP ^a	33	28	17
DB ^b	0.07	0.063	0.015
DP $\times$ DB ^c	2.3	1.7	0.26

724 ^aDP – degree of polymerization, ^bDB – degree of branching, ^cnumber of branches per molecule

Figures

Figure 1: Overview of carbohydrate pools resolved by an integrative MBTH method.

Insoluble sugars; Soluble, reducing sugars; and Soluble, total sugars are resolved by a warm water extraction, selective hydrolysis, and quantification with the MBTH reducing sugar assay. Compositionally, these three fractions represent structural carbohydrates, monosaccharides, and monosaccharides plus polysaccharides, respectively. The polysaccharide fraction (soluble, nonreducing carbohydrates) is determined by subtracting the soluble, reducing concentration from the soluble, total concentration.

Figure 2: Hexose composition analyses of laminarin and soluble glucan extracted from *Phaeodactylum tricornutum*. Gas chromatography traces of the neutral sugar standard (A), laminarin (B), and soluble glucan extracted from *Phaeodactylum tricornutum* (C). The neutral sugar standard contains the internal standard 3-*O*-methylglucose (1), myo-inositol (2), mannose (3), glucose (4), and galactose (5).

Figure 3: ^1H -NMR structural analysis of soluble polysaccharide extracted from *Phaeodactylum tricornutum*. A reference model of a β -glucan with labeled anhydroglucose units (AGU) whose unique ^1H correspond to previously annotated chemical shifts (A). The ^1H -NMR spectra of laminarin (B) and the purified, soluble polysaccharide from *Phaeodactylum tricornutum* (C) in DMSO-d_6 at 80°C are also presented. The major glucose doublet is the backbone chain (BC) and minor peaks include reducing terminal (RT, either α or β anomer),

747 second AGU adjacent to the reducing terminal (SRT), nonreducing terminal (NRT), side chain
748 (SC), and the terminal AGU of the SC (TSC).

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750 **Figure 4: Average carbohydrate fraction quantities extracted from *Phaeodactylum***

751 ***tricornutum* at dawn and dusk.** (n = 3, error = standard deviation; Paired student's T-test, * = p

752 < 0.05).

Figure 5: Cell physiology changes during nitrate deprivation. Cell density (A), cell size approximated by mean forward scatter per cell (B), total organic carbon (C), total nitrogen (D), F_v/F_m (E), chlorophyll *a* (F), and the chlorophyll *c* : chlorophyll *a* ratio (G) are depicted above. Black and white bars at the top of the figure represents day and night, respectively. Different letters represent statistically different groups from a Tukey's HSD post-hoc test after a repeated

759 measures one way ANOVA ($n = 5$, error = standard deviation, error bars may be smaller than
760 symbol).

Figure 6: Quantifying carbohydrate fractions and neutral lipid during nitrate deprivation.

The sum of all quantified carbohydrate fractions (A) is shown alongside soluble, nonreducing (B), insoluble (C), and soluble, reducing (D), carbohydrates and are reported as glucose equivalents (g.e.). Mean BODIPY fluorescence (E) and triacylglycerol (F) in tripalmitin equivalents (t.e.) are also reported. Black and white bars at the top of the figure represent day and night, respectively. Different letters represent statistically different groups from a Tukey's HSD post-hoc test after a repeated measures one way ANOVA ($n = 3-5$, error = standard deviation, error bars may be smaller than symbol).

Figure 7: Carbon partitioning into storage metabolites during nitrate deprivation. Black and white bars at the top of the figure represent day and night, respectively. Total carbon per cell is included as a reference (circles) for the total amount of carbon contributed by the storage metabolites triacylglycerol (squares) and soluble, nonreducing carbohydrate (inverted triangles). Mean triacylglycerol and soluble, nonreducing carbohydrate per cell were multiplied by carbon mass fractions for tripalmitin ($C_{51}H_{98}O_6$; 0.76) and glucose ($C_6H_{12}O_6$; 0.4) ($n = 3-5$, error = standard deviation, error bars may be smaller than symbol).