Quantitative label-free proteomics and biochemical analysis of *Phaeodactylum tricornutum* cultivation on dairy manure wastewater

Andrew R. Burch^{1,2} · Cody W. Yothers¹ · Michelle R. Salemi³ · Brett S. Phinney³ · Pramod Pandey^{4,5} · Annaliese K. Franz^{1,2}

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Abstract

Microalgae cultivation on wastewater offers the dual benefit of lowering costs for feedstock production with simultaneous wastewater remediation. This study utilized biochemical and quantitative label-free proteomic approaches to evaluate the growth and proteomic response for diatom *Phaeodactylum tricornutum* cultivated on flushed dairy manure wastewater (DMW). Comparing several DMW dilutions (up to 60% DMW diluted in seawater) with a synthetic seawater medium indicates that biomass and lipid yields correlate with the starting nitrogen content of the DMW dilution. *Phaeodactylum tricornutum* cultivated on DMW exhibits elevated levels of polyunsaturated fatty acids (PUFAs), particularly docosapentaenoic acid (DPA, 22:5 n-3). Proteomic analysis revealed alterations in the regulations of proteins associated with protein metabolism, cellular signaling, transcription and translation, protein trafficking, and oxidative stress management pathways when comparing *P. tricornutum* cultivation on diluted DMW versus synthetic media, thus providing insights into how *P. tricornutum* reorganizes its proteome in response to a complex wastewater source.

Keywords *Phaeodactylum tricornutum* · Quantitative proteomics · Microalgae · Wastewater · Dairy manure · Microbial lipids

Introduction

Microalgal lipids are a promising renewable feedstock for the production of biodiesel and other high value co-products. Microalgae can grow in aquatic environments with varying

salinities, possess higher growth and overall lipid production rates when compared to their terrestrial biofuel feedstock crop counterparts, and avoid conflicts over the use of terrestrial resources for fuel versus food production (Hannon et al. 2010). Despite these advantages, algae-derived biofuels are

✉ Annaliese K. Franz
akfranz@ucdavis.edu

¹ Department of Chemistry, University of California, Davis, CA 95616, USA

² Biochemistry, Molecular, Cellular and Developmental Biology Graduate Group, University of California, Davis, CA 95616, USA

³ Proteomics Core Facility, Genome Center, University of California, Davis, CA 95616, USA

⁴ Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis, CA 95616, USA

⁵ Division of Agriculture and Natural Resources, University of California Cooperative Extension, Davis, CA 95616, USA

currently not competitive compared to fossil fuels, partly due to the high costs associated with nutrient and water use for cultivating algae biomass (Fargione et al. 2008). Generating 1 kg of algae-derived biofuels requires 3000 kg of water, 0.33 kg of nitrogen, and 0.71 kg of phosphate when nutrients and water are not recycled (Jiang et al. 2011). The large nutrient and water input required for microalgae-based biofuel production suggest the importance of finding alternative nutrient and water sources for microalgae cultivation.

The use of wastewater sources such as flushed dairy manure wastewater (DMW) for algal biomass production is a viable strategy for reducing microalgae cultivation costs while also having potential benefit to the dairy industry (Chokshi et al. 2016). Microalgae can utilize inorganic nutrients present in DMW for growth and cellular processes, with nitrogen and phosphorus concentrations of DMW reaching 9.2–24.3 g kg⁻¹ and 1.2–4.1 g kg⁻¹ dry weight, respectively (Moreno-Caselles et al. 2002). With over 20 million tonnes per year of dairy manure produced yearly by dairy farms nationwide (Smith et al. 2016), unused manure must be managed to reduce negative environmental impacts such as surface water eutrophication and aquifer contamination. In general, DMW is applied as a crop fertilizer and in lesser amounts as a substrate for biogas production via anaerobic digestion (Demirer and Chen 2005). Additional opportunities exist to further exploit the use of DMW as an alternate nutrient source for microalgae biomass production.

Previous reports have demonstrated successful cultivation of microalgae species on a variety of wastewater sources (for review, see Rawat et al. 2016), but little is known about the molecular responses associated with microalgae cultivation in wastewater. “Omics” technologies represent an important option to investigate how microalgae respond to various wastewater cultivation practices, yet there is only one report of an “omic” approach to investigate microalgae responses to cultivation on wastewater. Patel and coworkers have reported the use of MS-based proteomics to characterize changes in the proteome of *Chlamydomonas reinhardtii* following cultivation on a synthetic wastewater medium (Patel et al. 2015). Previously, the use of “omic” technologies has also been employed to investigate microalgae responses to various alterations in environmental conditions, including nitrogen deprivation (Park et al. 2015), presence of heavy metals (Simon et al. 2013), and high saline concentrations (Ho et al. 2017).

Herein, we present the first example using proteomic analysis for evaluating the molecular response of microalgae cultured on an authentic (non-artificial) wastewater source. We use label-free quantitative proteomics combined with biochemical and physiological analyses to investigate the response of the marine diatom *Phaeodactylum tricornutum* cultivated on a seawater-diluted flushed DMW source, first by determining optimal DMW concentrations for comparison to synthetic media and then employing proteomics.

Phaeodactylum tricornutum was selected for this study based on its high lipid content, sequenced and annotated reference genome (Bowler et al. 2008; Michele Fabris et al. 2012), and documented ability to grow on wastewater sources (Coppens et al. 2014; Libralato et al. 2016). This study highlights the utility of DMW as an alternative nutrient source for microalgae cultivation and provides insights into how *P. tricornutum* reorganizes its proteome in response to cultivation on a complex wastewater source.

Material and methods

Flushed dairy manure wastewater collection, filtration, and characterization

Flushed DMW was collected from a manure storage lagoon located in Merced County of California, USA. Prior to entering the storage lagoon, flushed dairy manure was passed through a solid separator, which separates flushed manure into liquid and solid manure streams. The liquid manure stream was stored in a lagoon. Once collected from the lagoon and transported, the DMW was stored at 4 °C prior to use. Prior to its preparation as a culturing medium, the DMW was passed through a Wypall filter (Kimberly Clark, USA) and centrifuged to remove large suspended solids, producing a coarse-filtered DMW solution. Coarse-filtered DMW was subsequently sterilized by filtering through a 0.22-μm filter (Merck Millipore, USA) to produce the sterile-filtered DMW solution that was used throughout the study. Samples of both unfiltered and sterile-filtered DMW were analyzed by Denele Analytical, Inc. (Woodland, California, USA) using their Complete Lagoon Water Analysis package. For use as a culturing medium, DMW was diluted to specified dilutions using autoclaved, sterile-filtered seawater collected from the UC Davis-Bodega Marine Laboratory (Bodega Bay, CA, USA).

Microalgae culture maintenance on synthetic medium

An axenic culture of *P. tricornutum* (UTEX 466) was obtained from UTEX (University of Texas, Austin, TX, USA) and maintained on agar plates made of f/2 (F2) medium (NCMA, East Boothbay, ME, USA). Prior to inoculation into DMW dilutions as growth medium, *P. tricornutum* colonies from agar plates were inoculated into F2 liquid culture in 500-mL Corning Pyrex media bottles (Corning 1395-2 L) using stir bars with air bubbling (Petco Air Pump). During algae cultivation, incoming air was sterile-filtered by Polyvent 4 disposable filters (Whatman, UK). Culture suspensions were maintained at a constant temperature of 23 ± 2 °C with full spectrum incident uniform lighting (High Efficiency T-5 Grow Lights, Gardeners Supply Co, VT, USA) at a 16:8 h light/

dark cycle (60–120 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). All equipment and materials were autoclaved and media added to flasks in a Labconco Purifier Vertical Clean Bench (Labconco, USA) using sterile technique practices. Cell density was measured every other day at 680 nm using a Thermo Scientific Genesys 10S Vis Spectrophotometer (Thermo Fisher, USA).

Microalgae cultivation on sterile-filtered dairy manure wastewater

Liquid stock cultures of *P. tricornutum* were grown to a mid-logarithmic growth phase based on absorbance measurements at 680 nm. Prior to inoculation, cell counts on algae stock cultures were performed using an Improved Neubauer Hemocytometer (Hausser Scientific, USA) and inverted microscope (Eclipse TS100, Nikon, Japan). Algae stock solutions were inoculated into DMW dilutions at a 1:9 inoculant/DMW dilution ratio. All experiments were performed using 3–5 biological replicates and compared to control cultures inoculated into synthetic medium (F2). Throughout the experiment, growth was monitored by cell counting as described above.

Chlorophyll *a* determination

The chlorophyll *a* content of samples was measured by extraction using similar methods described by Ritchie (2006). Briefly, 1.0 mL of culture volumes was pelleted by centrifugation, washed with distilled H_2O (dH_2O), and re-pelleted. Following removal of the supernatant, microalgae pellets were flash frozen in liquid nitrogen and extracted in 1 mL of ice-cold methanol (100%, ACS grade). Chlorophyll *a* content was determined spectrophotometrically using the equation: chlorophyll *a* ($\mu\text{g mL}^{-1}$) = $12.9447 \times (\text{Abs}_{665} - \text{Abs}_{720})$.

Biomass determination

At the end of the experiment, microalgae cultures (250–300 mL) were pelleted by centrifugation and washed once with dH_2O . Algae pellets were then frozen in liquid nitrogen and lyophilized. Lyophilized biomass weights were recorded and are reported as milligram of harvested biomass per liter culture (mg L^{-1}). Lyophilized biomass was stored at -20°C under argon gas until utilized for lipid extraction assays as described below.

Lipid extraction

Total lipids were extracted from freeze-dried algae biomass using a modified Folch method (Folch et al. 1957). Microalgae samples were first treated with chloroform and pulverized using a hand sonicator (Fisher Scientific Model 120 Sonic Dismembrator, Thermo Fisher, USA) for 1 min.

Next, a 2:1 mixture of methanol/chloroform was added to samples and sonication repeated. Then, 0.1 M PBS buffer was added to samples, mixed vigorously, and centrifuged to separate layers. The lower organic (chloroform) phase containing lipids was removed and stored at -20°C .

Neutral lipid content determination using a Nile Red live cell assay

Intracellular lipid analysis of live *P. tricornutum* cultures was performed in a 96-well microplate as described by Franz and colleagues with slight modifications (Franz et al. 2013). Briefly, 25 μL of a 1:1 (v/v) DMSO/filtered seawater solution was added to 250 μL *P. tricornutum*, followed by addition of 4 μL of 1 mg mL^{-1} Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one, Sigma, USA) dissolved in acetone. Following a 5-min incubation in the dark, fluorescence measurements were captured using a Synergy HI Multi-Mode Plate Reader (Biotek, USA) at excitation and emission wavelengths of 530/40 nm and 590/40 nm, respectively. Background fluorescent measurements obtained from DMW dilution blanks were subtracted from wells prior to analysis.

Neutral lipid content determination using a bleach-enhanced Nile Red microplate lipid extract assay

A bleach-enhanced Nile Red microplate assay was adapted and used to quantify the amount of neutral lipids in the total lipid extract from microalgae (Higgins et al. 2014). This quantitative assay is referred to as a Nile Red neutral lipid assay and has been previously described by Burch and Franz (2016).

Lipid extraction and conversion to fatty acid methyl esters (FAMES)

Lyophilized *P. tricornutum* biomass (40–50 mg) samples were spiked with 1 μmol of an internal standard, heptadecanoic acid (Nu-check Prep, Inc., USA) dissolved in 100 μL of chloroform. To each sample, 400 μL of toluene, 3 mL of methanol, and 600 μL of 8% HCl in methanol solution were added. Samples were capped and incubated at 90°C for 60 min and then allowed to cool for 10 min. After cooling, 1 mL of hexane and then 1 mL of water were added to each sample to quench the reaction. Samples were vortexed and allowed to separate into organic/aqueous layers for 15 min, and complete separation of layers was attained by centrifugation. The upper hexane layer was withdrawn and washed with water, and then the remaining cellular debris was removed by centrifugation. FAME samples were stored at -80°C until analysis (Ichihara and Fukubayashi 2010).

Analysis of FAMES by gas chromatography

FAMES were analyzed using an Agilent 3800 gas chromatography system with a DB-23 fused silica column (0.25 mm ID $\times$ 30 m L, 0.25 mm film thickness; Agilent). Oven temperature was initially held at 50 °C for 2 min, ramped at 10 °C min⁻¹ to 180 °C, held for 5 min, ramped to 240 °C at 5 °C min⁻¹, and held for 5 min. Injector and detector temperatures were held at 250 °C and 300 °C, respectively. Helium was used as the carrier gas (1.3 mL min⁻¹) and inlet makeup gas. FAMES were identified by retention time using a mix of 31 FAME standards (NuChek Prep, Inc., USA), and FAME quantification was calculated by peak area relative to the internal standard as previously described (Ichihara and Fukubayashi 2010). Unidentified peaks were excluded from analysis.

Proteomic sample preparation

Phaeodactylum tricornutum samples were collected for label-free quantitative proteomic following 5 days of cultivation on DMW dilutions or F2 medium. Briefly, 10 mL of algae was pelleted by centrifugation and washed with sterile-filtered seawater. Following a second centrifugation step, washed algae pellets were flash frozen in liquid nitrogen and stored at - 80 °C until processed for proteomic analysis. Five biological replicates per condition (synthetic medium, 10% DMW, 20% DMW) were used for proteomic analysis ($n = 5$).

Thawed algae pellets were resuspended in RIPA buffer (Thermo Fisher Scientific, USA) containing protease inhibitors (Roche cOmplete ULTRA Tablets, Sigma Aldrich, USA) and lysed by mechanical disruption using a MagNA Lyser (Roche Diagnostics, USA). A 100 μ g of protein from each sample was precipitated using Calbiochem ProteoExtract Protein Precipitation Kit (Merck Millipore, USA). Precipitated proteins were resuspended into a 50 mM NH₃HCO₃ buffer containing 6.0 M urea and then reduced and subsequently alkylated using dithiothreitol (DTT) and iodoacetamide (IAA), respectively. Enzymatic digestion of proteins was performed using MS-grade LysC (Wako Chemicals USA) for 4 h at 37 °C and then diluted to reduce urea concentration and allowed to further digest overnight with sequencing grade modified trypsin (Promega, USA). After digestion, protein samples were desalted using a Macrospin microSPE kit (Nest Group, USA).

Digested peptide samples were analyzed by LC-MS/MS at the UC Davis Proteomic Core Facility using a Thermo Scientific Q Exactive Plus Orbitrap Mass Spectrometer in conjunction with a Proxeon Easy-nLC II HPLC and Proxeon nanospray source. The digested peptides were loaded on a 100 μ m $\times$ 25 mm Magic C18 100

Å 5 U reverse phase trap where they were desalted online before being separated using a 75 μ m $\times$ 150 mm Magic C18 200 Å 3 U reverse phase column. Peptides were eluted using a 120-min gradient (2–35% buffer B for 85 min, 35–80% buffer B for 25 min, 2% buffer B for 10 min; buffer A = 0.01% formic acid in H₂O, buffer B = 100% acetonitrile) with a flow rate of 300 nL min⁻¹. An MS survey scan was obtained for the m/z range 300–1600, and MS/MS spectra were acquired using a top 15 method, where the top 15 ions in the MS spectra were subjected to high energy collisional dissociation (HCD). An isolation mass window of 1.6 m/z was used for the precursor ion selection, with a normalized collision energy of 27% for fragmentation. Fifteen seconds was used for the dynamic exclusion.

Proteomic data analysis

Peptide spectral matching (PSM) and protein identification was performed using MaxQuant (v. 1.5.7.4) against the UniProt reference proteome for *P. tricornutum* with the default MaxQuant laboratory contaminant sequences (Apweiler et al. 2004). Decoy false discovery rates (FDR) were limited to less than 1% at the peptide and protein level. Identified proteins were considered for subsequent downstream analysis only if they were identified in at least three biological replicates in at least one experimental condition. The Perseus (v. 1.5.6.0) computation platform was used for statistical analysis, and a one-way ANOVA was performed using a permutation-based FDR strategy (FDR > 0.05) at a significance level of 5%. Subsequent post hoc analysis (Tukey's HSD) and two-tailed t -tests were performed to identify and determine fold change differences in significant ($p < 0.05$) proteins across conditions (F2, 10% DMW, 20% DMW). Proteins were utilized in downstream analysis only if the observed fold change between conditions was greater than 2-fold ($\log_2\text{FC} \pm 1$) and p value < 0.05. In this study, in-text fold changes ($\log_2\text{FC} \pm 1$ and p value < 0.05) are reported after UniProt IDs (e.g., UniProt ID, 10%/20% + 2.0), with the percentage values indicating the conditions being compared. Functional annotations, including gene ontology (GO), pfam, and interpro annotations for each observed significant protein, were obtained from the UniProt website. Additional annotation was performed using the Mercator webserver (Lohse et al. 2014) followed by manual curation using DiatomCyc (Fabris et al. 2012), KEGG (Kanehisa and Goto 2000), and JGI databases (Nordberg et al. 2013). Visualization of data was performed using MapMan software (Thimm et al. 2004), the KEGG Mapper “Search&Color Pathway” tool (Kanehisa et al. 2012), and JColorGrid (Joachimciak et al. 2006).

Microscopy evaluation of *P. tricornutum* grown on dairy manure wastewater

Microscopic analysis and imaging of *P. tricornutum* grown on F2 and DMW dilutions were performed at multiple time points throughout the culture period using an inverted light microscope (Nikon TS100 Eclipse). Images were acquired using a mounted camera (Canon EOS Rebel XS) in combination with a $\times 100$ oil immersion lens (Nikon CFI Achro 100X 1.4 NA). Images were processed using ImageJ version 1.52a (Schneider et al. 2012).

Total nitrogen content analysis

The total nitrogen content of F2 and DMW media sources were determined by a persulfate digestion method using a Hach Total Nitrogen Test N' Tube Reagent Set (Hach, USA) according to the manufacturer's recommendations.

Iron analysis of F2 and DMW media sources

Iron content of F2 and DMW media sources were determined by the UC Davis Analytical Laboratory using a nitric acid/hydrogen peroxide microwave digestion procedure followed by determination via atomic absorption spectrometry and inductively coupled plasma (ICP) atomic emission spectrometry.

Results

Analysis of dairy wastewater (DMW) composition

Synthetic media (F2), DMW, and sterile-filtered DMW were analyzed by Denele Analytical, Inc. (Woodland, California)

using the Complete Lagoon Water Analysis package (Table 1). Sterile filtration of the DMW resulted in a notable reduction of total Kjeldahl nitrogen (TKN) from 123 to 56 mg L⁻¹ and phosphorous from 21.7 to 4.57 mg L⁻¹, resulting from volatilization and suspended solid exclusion respectively.

Cell density, biomass production, and chlorophyll *a* content of *P. tricornutum* cultivated using DMW

Phaeodactylum tricornutum cultivated on 10%, 30%, and 60% DMW for 15 days resulted in differences in cell density, biomass production, and chlorophyll *a* content when comparing DMW dilutions to each other and when compared to cultivation using synthetic medium (F2) over the same time period (Fig. 1a–c). Cell density after 15 days of culturing was similar in the F2, 30%, and 60% conditions, with all three conditions reaching between 4.97×10^6 and 5.24×10^6 cells mL⁻¹. Compared to the F2 control, increases in the chlorophyll *a* content were observed using 30% and 60% DMW conditions (Fig. 1b). In both the F2 and 30% DMW conditions, chlorophyll *a* content peaked on day 10 at 2.6 $\mu\text{g mL}^{-1}$ and 3.3 $\mu\text{g mL}^{-1}$, respectively, and declined to 2.0 $\mu\text{g mL}^{-1}$ and 2.7 $\mu\text{g mL}^{-1}$ by day 15, respectively.

No decrease in chlorophyll *a* content was observed through the growth period in the 60% DMW condition, with the chlorophyll *a* content reaching 4.6 $\mu\text{g mL}^{-1}$ by day 15. A similar pattern was observed in biomass harvested at day 15 (Fig. 1c), with F2, 30%, and 60% DMW yielding approximately 309 mg L⁻¹, 569 mg L⁻¹, and 774 mg L⁻¹ dried biomass, respectively. A reduction in cell density was observed in the 10% DMW condition by day 15 compared to all other conditions, with cultures reaching approximately 2.34×10^6 cells mL⁻¹. Similarly, *P. tricornutum* cultivated using 10% DMW had a lower chlorophyll *a* content throughout the culture period, peaking at 1.0 $\mu\text{g chlorophyll } a$ per mL at day 3, with

Table 1 Water analysis of the synthetic media (F2) and unfiltered and filtered flushed dairy manure wastewater source (DMW)

Analyte	F2 synthetic media	Unfiltered DMW	Sterile-filtered DMW	Units	Method reference
pH	8.49	8.16	9.02	units	EPA 150.2
EC	54.3	5.34	6.01	mmhos cm ⁻¹	EPA 120.1
Ca	314	59.3	18.6	mg L ⁻¹	EPA 200.7
Mg	1010	126	147	mg L ⁻¹	EPA 200.7
Na	9880	389	474	mg L ⁻¹	EPA 200.7
K	321	726	976	mg L ⁻¹	EPA 200.7
B	3.60	0.191	0.233	mg L ⁻¹	EPA 200.7
NO ₃ -N	14.8	79.3	20.9	mg L ⁻¹	EPA 300.0
TKN	39.2	123	56	mg L ⁻¹	SM 4500-NH ₃ C
NH ₃ -N	<0.50	8.4	<0.50	mg L ⁻¹	SM 4500-NH ₃ C
P	0.731	21.7	4.57	mg L ⁻¹	SM 4500P-E

Fig. 1 **a** Growth of *P. tricornutum* on synthetic medium (F2) or 10%, 30%, and 60% DMW dilutions over a 15-day period as measured by cell density, **b** chlorophyll *a* content, and **c** total harvested biomass (day 15). **c** Comparison of total biomass harvested on day 15 (primary y-axis) compared to the starting nitrogen content of diluted DMW versus synthetic medium (secondary y-axis). Data shown (**a–c**) as mean $\pm$ SD ($n = 3$). **d** Microscopic images ($\times 100$) of *P. tricornutum* cultured on F2 or DMW dilutions on day 15 of the 15-day growth period

less biomass (247 mg L⁻¹) harvested by day 15 when compared to all other conditions.

Neutral lipid content of *P. tricornutum* grown on DMW dilutions

Differences in the neutral lipid content of *P. tricornutum* grown on 10%, 30%, and 60% were observed when compared to cultures grown on F2 medium using two Nile Red staining methods (Fig. 2a,b). The differences in neutral lipid content of harvested algae biomass were confirmed at day 15 using an additional Nile Red assay (Burch and Franz 2016; Higgins et al. 2014) that compared the Nile Red fluorescence of lipid extracts against a known oil standard (Fig. 2b). An increase in the neutral lipid content was observed in the 10% DMW

condition (0.36 mg neutral lipids per mg biomass (mg mg⁻¹)) when compared to synthetic medium (F2) control (0.15 mg mg⁻¹), 30% DMW (0.10 mg mg⁻¹), and 60% DMW (0.034 mg mg⁻¹).

Fatty acid profile of *P. tricornutum* grown on DMW dilutions

Cultivation of *P. tricornutum* on increasing concentrations of DMW generally decreased the levels of saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) while increasing docosapentaenoic acid (DPA, C22:5 n-6) relative to cultivation in synthetic medium (Table 2, Supplementary Table 1). Cultivation using 30% and 60% DMW shifted the

Fig. 2 **a** Neutral lipid content of *P. tricornutum* grown on synthetic medium (F2) or 10%, 30%, and 60% DMW dilutions as measured by in vivo Nile Red fluorescence. **b** Comparison of the neutral lipid content per biomass (mg mg⁻¹) of day 15 crude lipid extracts (primary y-axis) compared to the starting nitrogen content of diluted DMW versus synthetic medium (secondary y-axis). Data shown as mean $\pm$ SD ($n = 3$)

fatty acid profile where an increase in DPA content was especially notable compared to F2 and 10% DMW conditions.

Overview of proteomic data comparing *P. tricornutum* growth on DMW dilutions to growth on synthetic medium

Phaeodactylum tricornutum was grown on either 10% DMW (nitrogen-limited), 20% DMW (nitrogen-equivalent), or synthetic medium (F2) and sampled for proteomic analysis after 5 days of cultivation. Using a label-free quantitative approach, a total of 1562 proteins were identified across all conditions, and an analysis of variance (ANOVA) test identified 268 proteins with significantly different ($p < 0.05$) expression patterns across all conditions. Post hoc analysis (Tukey's HSD) and pairwise comparison of significantly different proteins across conditions (10% DMW-F2, 20% DMW-F2, and 10% DMW-20% DMW) identified 198, 104, and 102 proteins with significantly different expression across conditions, respectively (Fig. 3a,b). Fold change expression of significantly ($p < 0.05$,

$\log_2\text{FC} \pm 1$) regulated proteins compared to each condition are reflected in Fig. 4 and categorized into nitrogen metabolism (Fig. 4a), iron limitation (Fig. 4b), photosynthesis-related pathways (Fig. 4c), central carbon metabolism (Fig. 4d), redox-related pathways (Fig. 4e), and gene expression and signaling (Fig. 4f).

Discussion

Cell density, biomass production and chlorophyll *a* content of *P. tricornutum* cultivated using DMW

The difference in *P. tricornutum* harvested biomass and chlorophyll *a* content observed across conditions is attributed to variation in the nitrogen content of the diluted DMW and synthetic media sources used throughout the study. The 10%, 30%, and 60% DMW dilutions contain approximately -49%, 35%, and 244% of the nitrogen present in the synthetic medium, respectively (Fig. 1c). A positive relationship between nitrogen content, chlorophyll content, and growth has been observed across all major evolutionary lineages of microalgae including *P. tricornutum* and other diatom species (Qiao et al. 2016; Yodsuan et al. 2017). Nitrogen limitation reduces the cellular chlorophyll content in *P. tricornutum* (Valenzuela et al. 2013) and is considered part of a nitrogen-recycling mechanism that repurposes the nitrogen contained within the chlorophyll porphyrin rings and chlorophyll binding proteins for other cellular processes (Hörtensteiner and Kräutler 2011). Discrepancies observed between cell density, chlorophyll *a* content, and biomass measurements (Fig. 1a–c) in the 30% and 60% DMW conditions compared to the F2 control may be in part due to the complex composition of the dairy wastewater medium source. The increase in biomass and chlorophyll *a* content observed following cultivation on 30% and 60% DMW dilutions is consistent with the higher starting nitrogen content in these conditions compared to the F2 control, yet no difference in the final cell density was observed at the end of the culture period. Microscopic observation of cultures throughout the culture period (Fig. 1d) rules out microbial contamination but revealed the presence of cellular debris in the 30% and 60% DMW conditions compared to the F2 and 10% DMW conditions. Therefore, these results present the possibility that an unidentified component of the wastewater medium may promote cell degradation in the higher DMW concentrations. These results informed our design of the proteomic experiments to include a “nitrogen equivalent” dilution of DMW compared to the F2 media (20%), as well as a “nitrogen deficient” dilution (10%).

Table 2 Fatty acid composition (% of total fatty acids) of *P. tricornutum* cultivated on DMW dilutions and F2 synthetic medium for 15 days ($n = 3$)

	F2			10% DMW			30% DMW			60% DMW		
C14:0	6.97	±	0.7	6.21	±	0.07	8.64	±	0.7	10.92	±	0.3
C16:0	20.81	±	3.0	24.69	±	1.5	17.14	±	1.9	13.68	±	1.4
C16:1	42.49	±	2.1	42.70	±	1.4	29.59	±	2.3	21.76	±	1.1
C18:0	0.67	±	0.04	1.06	±	0.06	0.77	±	0.1	0.24	±	0.3
C18:1 cis	3.17	±	1.7	4.34	±	0.9	4.11	±	1.2	2.85	±	0.2
C18:2 n-6	0.60	±	0.01	0.37	±	0.03	0.41	±	0.2	1.13	±	0.1
C18:3 n-6	0.84	±	0.07	0.95	±	0.1	0.53	±	0.3	n.d.		
C20:2 n-6	0.32	±	0.03	0.93	±	0.1	1.20	±	0.2	n.d.		
C20:4 n-6	0.58	±	0.2	0.66	±	0.2	0.64	±	0.4	n.d.		
C20:5 n-3	19.35	±	1.1	8.61	±	0.5	19.56	±	1.4	30.47	±	1.1
C22:2	1.49	±	0.2	5.13	±	0.6	4.66	±	0.7	0.96	±	0.1
C22:5n-6	1.51	±	0.2	3.72	±	1.1	12.46	±	2.1	17.54	±	1.1
C22:6	0.93	±	0.1	0.45	±	0.1	0.30	±	0.3	0.43	±	0.5
TFA (%)	71.84	±	9.6	71.83	±	6.8	60.17	±	11.7	61.58	±	6.0
Saturated	20.61	±	3.8	22.88	±	1.7	15.96	±	2.7	15.34	±	1.9
MUFA	32.77	±	3.8	33.71	±	2.3	20.27	±	3.6	15.16	±	1.3
PUFA	18.46	±	2.0	15.24	±	2.8	23.94	±	5.5	31.08	±	2.8
C16:1/16:0	2.05			1.75			1.73			1.59		
C18:1/18:0	4.69			4.00			5.31			10.71		
18 u.s./18:0	6.86			5.28			6.53			15.01		
Total u.s./total sat.	2.49			2.14			2.77			3.01		

u.s. mono and poly unsaturated, MUFA monounsaturated fatty acid, PUFA polyunsaturated fatty acid. Values are represented as mean ± standard deviation

Neutral lipid content of *P. tricornutum* grown on DMW dilutions

Differences in the neutral lipid content of *P. tricornutum* grown on 10%, 30%, and 60% were observed when compared to cultures grown on F2 medium using two Nile Red staining methods (Fig. 2a,b). The first Nile Red method (Chen et al. 2009; Franz et al. 2013) measured the relative intracellular lipid content throughout the 15-day culture period and revealed an increase in the relative intracellular lipid content of 10% DMW compared to all other conditions by day 10 (Fig. 2a). The neutral lipid content of *P. tricornutum* cultured in the 60% DMW condition remained relatively unchanged over time, whereas the neutral lipid content gradually increased throughout the culture period in the F2, 10%, and 30% DMW conditions. An increase in the neutral lipid content was observed in the 10% DMW condition (0.36 mg neutral lipids/mg biomass (mg mg^{-1})) when compared to synthetic medium (F2) control (0.15 mg mg^{-1}), 30% DMW (0.10 mg mg^{-1}), and 60% DMW (0.034 mg mg^{-1}) (Fig. 2b). The increase in neutral lipid content following cultivation on the 10% DMW condition is most likely a response to nitrogen depletion conditions (Burch and Franz 2016; Yang et al. 2014; Yodsuwan et al. 2017) and is supported by the

differences in growth, biomass, chlorophyll *a* content, and initial nitrogen concentrations detected between the conditions tested (Fig. 1a–c, Fig. 2b).

Fatty acid profile of *P. tricornutum* grown on DMW dilutions

Cultivation of *P. tricornutum* using more DMW generally decreased the levels of saturated fatty acids (SFAs) and mono-unsaturated fatty acids (MUFAs) while increasing docosapentaenoic acid (DPA, C22:5 n-6) relative to cultivation in synthetic medium (Table 2, Supplementary Table 1). Consistent with previous reports for *P. tricornutum* and other diatoms (Siron et al. 1989; Yang et al. 2017; Zhao et al. 2014), fatty acid analysis using GC-MS revealed that palmitic acid (16:0), palmitoleic acid (16:1), and eicosapentaenoic acid (EPA, 20:5 n-3) were the most abundant species detected when using 10% and 30% DMW for cultivation. In addition, the relative abundance of DPA to DHA is consistent with previous reports (Yang et al. 2017). Cultivation using 30% and 60% DMW shifted the fatty acid profile where an increase in DPA content was especially notable compared to F2 and 10% DMW conditions.

Fig. 3 **a** Volcano plots of proteins showing fold change and statistical significance of change across comparison conditions. Significantly ($p < 0.05$, $\log_2FC \geq 1$) regulated proteins are represented by red triangles in volcano plots. **b** Bar charts of proteins showing significant upregulation (green) and downregulation (red) across comparison conditions. **Post hoc comparisons using Tukey's HSD test followed by independent t -tests where $\log_2FC \geq 1$

This shift to produce more DPA using DMW correlates with the higher initial nitrogen content when more DMW is utilized for cultivation, but the combined data (Supplementary Table 1) suggest that nitrogen content is not the only factor contributing to this effect. *Phaeodactylum tricornutum* and other diatoms are known to increase SFA and MUFA levels in response to nutrient limitation (Liang et al. 2006; Liang and Mai 2005), and the modification of the initial nitrogen concentration in media has been shown to influence fatty acid composition in *P. tricornutum* (Qiao et al. 2016). The accumulation of DPA may also be a physiological response to stressors present in the DMW that enhances the resilience in *P. tricornutum*. As membrane fluidity increases with chain length and degree of unsaturation (Harwood 1988; Jiang and Gao 2004), DPA may contribute to the resilience of *P. tricornutum* through the relief of turgor pressure, protein recruitment, and nutrient uptake (for review, see Juneja et al. (2013) and Sayanova et al. (2017)). Given that DPA is a commercially valuable omega-3 fatty acid with well-documented human health benefits (Byelashov et al. 2015), increased DPA production using a cheap and abundant alternative nutrient source may have important commercial applications.

Overview of proteomic data comparing *P. tricornutum* growth on DMW dilutions to growth on synthetic medium

Biochemical and physiological analysis of *P. tricornutum* cultured in DMW revealed similarities to results observed following nitrogen limitation in this species, with increased lipid contents associated with lower environmental nitrogen concentrations. Therefore, proteomic analysis was performed on *P. tricornutum* cultures grown on two DMW concentrations that were either nitrogen-limited or nitrogen-equivalent when compared to the nitrogen content of synthetic medium. *P. tricornutum* was grown on either 10% DMW (nitrogen-limited), 20% DMW (nitrogen-equivalent), or synthetic medium (F2) and sampled for proteomic analysis after 5 days of cultivation (see the “Overview of proteomic data comparing *P. tricornutum* growth on DMW dilutions to growth on synthetic medium” section and Fig. 3a,b).

Nitrogen metabolism

Proteins involved in nitrogen metabolism were upregulated in the nitrogen-limited 10% DMW condition but not with nitrogen-equivalent 20% DMW compared to F2 (Fig. 4a). *P. tricornutum* responded to the low environmental nitrogen levels in 10% DMW by increasing its nitrogen uptake and assimilation abilities, as evidenced by the upregulation of a urea transporter (B7FZW5, 10%/F2 + 1.54), nitrate reductase (B7G997, 10%/F2 + 1.35) and glutamate dehydrogenase (B7G3X3, 10%/F2 + 1.30). Upregulation of a xanthine/uracil permease (B7FP05, 10%/F2 +

Fig. 4 Fold change expression of significantly ($p < 0.05$, $\log_2FC \pm 1$) regulated proteins involved in **a** nitrogen metabolism, **b** iron limitation, **c** photosynthesis-related pathways, **d** central carbon metabolism, **e** redox-related pathways, and **f** gene expression and signaling. Rows represent

proteins with unique UniProt ID identifier, and columns specify comparison condition. Blue and red colored boxes signify upregulation or down-regulation of abundance in each specific comparison, respectively. Grey boxes represent no significant difference across comparison conditions

4.07), purine permease (B7GBV6, 10%/F2 + 2.04), and xanthine dehydrogenase (B7GAV1, 10%/F2 + 1.28) may also provide *P. tricornutum* with an additional source of nitrogen containing compounds during nitrogen limitation. Cultivation on 10% DMW also led to upregulation of proteins whose functions include the release of ammonia from various macromolecules, including a formidase (B7FYS6, 10%/F2 + 1.89), a putative amidase (B7GE02 + 4.15), two putative cytidine deaminases (B7GCB8, 10%/F2 + 2.08, B7FYE0, 10%/F2 + 2.45), and a protein with hydrolase activity (B7G1X3, 10%/F2 + 2.22) involved in the NAD salvage pathway that catalyzes the release ammonia from nicotinamide. Collectively, upregulation of these proteins supports the conclusion of Longworth and colleagues that *P. tricornutum* cells remodel their proteome to internally scavenge and recycle nitrogen sources during nitrogen-limited conditions (Longworth et al. 2016).

Iron response

When compared to the F2 control, *P. tricornutum* grown on either 10% and 20% DMW conditions led to upregulation of multiple proteins associated with an iron limitation response (Fig. 4b) (Allen et al. 2008; Lommer et al. 2012; Morrissey et al. 2015; Morrissey and Bowler 2012). The upregulation of proteins associated with iron limitation suggested that the DMW study was lacking in iron. Subsequent analytical testing of the soluble iron content confirmed the iron-limited nature of the DMW, with the 20% and 10% DMW conditions containing only 3.4% (0.014 mg L⁻¹) and 1.7% (0.007 mg L⁻¹) of the iron found in the F2 medium (0.414 mg L⁻¹), respectively. Diatoms as a group have historically been utilized in environmental

monitoring studies (Dixit and Smol 1994) and can survive during periods of low environmental iron availability, with *P. tricornutum* being particularly tolerant to conditions of low environmental iron (Kustka et al. 2007). The response to low iron levels was especially dramatic in the 20% DMW condition, with four of the top five most upregulated proteins (9–16-fold) identified matching proteins previously linked to an iron starvation response (Allen et al. 2008). These highly induced proteins include iron starvation-induced protein ISIP2A (B7FYL2, 20%/F2 + 4.74), ISIP1 (B7GA90, 20%/F2 + 7.93), ISIP3 (B7G4H8, 20%/F2 + 6.25), and flavodoxin (B7GCM3, 20%/F2 + 6.52). ISIP2A is a cell surface protein whose function in the concentration and uptake of Fe(III) was recently characterized (Morrissey et al. 2015), whereas ISIP1 and ISIP3 are cell surface proteins whose functions during iron limitation remain to be determined. Flavodoxin is an electron-transfer protein that substitutes for ferredoxin in the photosynthetic electron transport during periods of iron limitation.

The expression patterns of iron-responsive proteins in *P. tricornutum* cultured on 10% DMW conditions suggests that the proteomic response is less robust in 10% DMW compared to 20% DMW condition. Expression of multiple iron response proteins were significantly higher in the 20% DMW condition (Fig. 4b), and the muted iron limitation response observed in the 10% DMW dataset may be in part due to *P. tricornutum* reacting to the additional stress of low environmental nitrogen that was not present in the 20% DMW condition. Other data from this study support this hypothesis, as *P. tricornutum* grown in 10% DMW showed a robust response to nitrogen limitation and most likely diverted substantial amounts of energy and resources to synthesize proteins

necessary for survival in this environment. These differences are attributed in part to temporal aspects of the iron response and are the result of when sampling occurred for proteomic analysis.

Amino acid metabolism

Differences in the abundance of several proteins involved with amino acid metabolism were observed in *P. tricornutum* cultures for both 10% and 20% DMW, but meaningful trends in the data were not observed. The upregulation of a vitamin B12-independent methionine synthase (B7G1X4, 10%/F2 + 3.38, 20%/F2 + 2.89) was observed in both 10% DMW and 20% DMW conditions, and this protein is upregulated in cobalamin-limited environments (Bertrand et al. 2012). Additional downregulation of a vitamin B12-dependent methionine synthase (B7GBG7, 10%/F2 -1.21) was observed in the 10% DMW condition. Taken together, differential regulation of these proteins indicates that this DMW source may also be limiting in other micronutrients besides iron.

Photosynthesis

Photosynthesis-related proteins were generally downregulated when comparing the nitrogen-deplete 10% DMW condition to both the nitrogen-equivalent 20% DMW and F2 conditions (Fig. 4c, Supplementary Fig. 1). The most prominent downregulation occurred in pathways controlling biosynthesis of photosynthetic pigments and their precursors. Downregulations of glutamyl-tRNA synthase (B7GCT6, 10%/F2 -1.69, 20%/10% -2.02) and hemL (B7G134, 10%/F2 -1.20) indicate a reduction in the biosynthesis of tetrapyrroles, compounds with important functions as cofactors in chlorophyll pigments, as well as heme-containing proteins. Decreased expressions of proteins hemE (B7GB77, 10%/F2 -1.74), PPO (B7GDU9, 10%/F2 -2.00, 10%/20% -1.67), CHLD (B7FTA2, 10%/F2 -1.75, 10%/20% -1.43), and DVR (B7GC47, 10%/F2 -1.75) indicate that chlorophyll biosynthesis was also downregulated. Biosynthesis of carotenoids was also downregulated, as evidenced by the downregulation of proteins involved in biosynthesis of isoprenoids (HDS, B7FV10 10%/F2 -1.26; GGPS, B7FU89, 10%/F2 -1.74, 10%/20%, -1.59) and carotenoids (PDS1, B7FZL9, 10%/F2 -1.48; ZDS, B7FPC4, 10%/F2 -1.18; CRTISO5, B7FQF7, 10%/20% -1.67). No evidence of downregulation of chlorophyll and carotenoid biosynthesis was observed when comparing the 20% DMW condition to the F2 control.

Multiple light-harvesting complex (LHCs) fucoxanthin-chlorophyll *a/c* binding proteins (FCPs) were observed upregulated in the 10% and 20% DMW conditions compared to the F2 control (Fig. 4c). Unlike what has been observed in other proteomic experiments investigating nitrogen limitation in *P. tricornutum*, cultivation in 10% DMW culture did not lead

to a general decrease in the expression of chlorophyll binding light-harvesting protein, and the pattern of regulation in these proteins resembles the 20% DMW condition. Iron deficiency has been shown to cause a rearrangement of the photosynthetic apparatus in diatoms (Allen et al. 2008) and may influence light-harvesting proteins in an effort to increase photoprotection mechanisms. Indeed, LHCX2 (B7FR60, 20%/F2 + 2.85, 10%/F2 + 2.51), an important antenna protein involved in nonphotochemical quenching (NPQ) in *P. tricornutum* (Lepetit et al. 2017; Nymark et al. 2009), was upregulated in both 10% and 20% DMW conditions. Flavodoxin (B7GCM3, 20%/F2 + 6.52, 10%/F2 + 6.70), as described above, was upregulated in both 10% and 20% DMW conditions when compared to the F2 control and was the only differentially regulated protein specific to the photosynthetic apparatus.

Rubisco activase (A0T0M5, 10%/F2 -1.14) and the carbonic anhydrase isoform CA4 (B7FNU0, 10%/F2 -1.92) were downregulated in the 10% DMW condition, suggesting the decreased role of photosynthetic carbon fixation during periods of nitrogen limitation. A candidate bicarbonate transporter part of the SLC4 family (B7S437, 20%/F2 -2.65, 10%/F2 -2.47) was also downregulated in both 10% and 20% DMW conditions. Finally, FBA5C (B7GE67, 20%/F2 + 4.66, 10%/F2 + 3.12), a plastidic fructose biphosphate aldolase isoform, was upregulated in both 10% and 20% DMW conditions. It has been hypothesized that during times of low carbon assimilation, a condition observed during iron limitation in *P. tricornutum* (Allen et al. 2008), the reaction FBAC5 catalyzes may run in the reverse direction, consequently supplying carbon skeletons for anabolic reactions (Allen et al. 2012).

Protein metabolism

After 5 days of cultivation on 10% and 20% DMW, differential regulation of multiple proteins involved in protein synthesis and degradation was observed (Fig. 5). Compared to the F2 control, *P. tricornutum* cultured on 10% DMW led to downregulation of 19 ribosomal structural proteins, indicating an overall decrease in protein synthesis. Four proteins with annotated proteolytic functions were upregulated (all 10%/F2: B7GDQ0, + 1.60; B7FSD0, + 3.25; B7FUY6, + 1.25; B7G5X7, + 2.36), while one protease (B5Y4I2, 10%/F2 -1.89) and a ubiquitin extension protein (B7G5H7, 10%/F2 -2.26) were downregulated. *P. tricornutum* grown in 20% DMW also exhibited mixed regulation of proteins involved in protein degradation with two proteases upregulated (all 20%/F2: B7FSD0, + 3.07; B7FVG8, + 1.73) and one protease downregulated (B5Y4I2, 20%/F2 -2.85). However, in contrast to the 10% DMW condition, the downregulation

Fig. 5 MapMan-based visualization of significantly ($p < 0.05$, $\log_2FC \pm 1$) regulated proteins involved in protein metabolism in **a** F2 compared to 10% DMW and **b** F2 compared to 20% DMW. Proteins are represented as individual blocks and colored blue or red if upregulated or downregulated in abundance, respectively

of ribosomal structural proteins was not observed, and five ribosomal structural proteins (all 20%/F2: A0T0J5, + 1.45; B7FP95, + 2.85; A0T0C7, + 1.80; A0T0I4, + 1.91; B7FQC3, + 2.22) were upregulated when compared to the F2 control.

Proteins involved in regulating protein synthesis, including those involved in translation initiation (B7G3T4, 20%/F2 + 1.58, 10%/F2 + 1.56; B7FV44, 20%/F2 2.23, 10%/F2 2.91) and elongation (B7G3C4, 20%/F2 + 1.53, 10%/F2 + 1.44), were upregulated in both 10% and 20% DMW conditions, with an additional protein with functions in translation initiation (B7G5G1, 10%/F2 + 1.78) upregulated in the 10% DMW condition only. Taken together, this regulation pattern supports the conclusion that *P. tricornutum* grown in 10% DMW decreases protein synthesis while simultaneously enhancing targeted protein degradation. This response may function as part of a mechanism that reduces protein synthesis as a general sink for nitrogen while also increasing nitrogen availability for the biosynthesis of specific proteins during periods of low environmental nitrogen availability. Organisms exert a tight control on protein synthesis and degradation in response to fluctuating environmental conditions, and the mixed regulation of proteases combined with the upregulation of ribosomal structural proteins and of proteins with translation initiation and elongation factor activity suggest the importance of controlling protein synthesis during cultivation on this DMW source.

Central carbon metabolism and oxidative phosphorylation

A broad upregulation of proteins involved in central carbon metabolism and oxidative phosphorylation were observed in *P. tricornutum* cultivated on 10% DMW, including enzymes associated with glycolysis (B7GC94, 10%/F2 + 1.27; B7G8B7, 10%/F2 + 1.73), the TCA cycle (all 10%/F2: B7FZD7, + 1.39; B7G228, + 1.27; B7FWX5, + 2.35), and oxidative phosphorylation (all 10%/F2 B7GBT9, + 2.14; B7FXG3, + 1.19; B7G964, + 2.11; B7G360, + 1.61) (Fig. 4d). Upregulation of dihydroorotate dehydrogenase (B7G8S8, + 1.91) during cultivation on 10% DMW was also observed, which functions in pyrimidine biosynthesis and energy generation at the mitochondrial membrane. The additional upregulation of two putative mitochondrial phosphate transporters observed following cultivation on 10% DMW (B7G6E4, 10%/F2 + 1.27) or 10% and 20% DMW conditions (B7G5H6, 20%/F2 + 1.86, 10%/F2 + 1.67), respectively, and may function to supply inorganic phosphate to the mitochondria for ATP biosynthesis.

Interestingly, cultivation on 10% and 20% DMW dilutions resulted in an upregulation of two short chain dehydrogenase/reductases (SDR) (B7G0Q2, 20%/F2 + 1.99, 10%/F2 + 1.54; B7GBR2, 20%/F2 + 2.11, 10%/F2 + 2.61) and an aldo-keto reductase (AKR) (B7GDN9, 20%/F2 + 1.78, 10%/F2 + 1.84) when compared to the F2 control. Although SDR and AKR protein families are very diverse in their structure and

functions, both families contain NAD⁺/NADP⁺ binding domains and can participate in oxidoreductase reactions that reduce aldehydes and ketones to primary and secondary alcohols, respectively (Penning 2015). The downregulation of glyoxalase II (B7GDI1, 20%/F2 -2.20) in the 20% DMW condition could mean that the observed upregulation of AKR and SDR proteins with unknown specific functions may be involved in the detoxification of methylglyoxal (Grant et al. 2003; Yamauchi et al. 2011), a toxic byproduct of carbon metabolism. SDR and AKR protein families also have known roles in redox sensing (Kavanagh et al. 2008) and stress tolerance (Kanayama et al. 2014), and three additional alcohol dehydrogenases (all 10%/F2: B7GEB0, + 1.44; B7GEA6, + 1.44; B7S4B2, + 1.35) of unknown specificity that were upregulated following 10% DMW cultivation may have been upregulated in response to low levels of environmental nitrogen.

Redox/stress

Analysis of the proteome suggests that *P. tricornutum* responds to DMW conditions by increasing the abilities to regulate oxidative stress levels and maintain redox homeostasis (Fig. 4e). The increased expression of glutathione reductase (GSR_1, B7G9Q1, 10%/F2 + 2.01) in 10% DMW and glutathione synthase (B5Y5J7, 20%/F2 + 1.78) in 20% DMW suggests that *P. tricornutum* attempts to increase or replenish reduced glutathione (GSH) levels in response to DMW conditions. Maintaining high levels of reduced glutathione pools have been shown to increase abiotic stress tolerance in *Arabidopsis* (Cheng et al. 2015) and may enhance the ROS detoxification ability of *P. tricornutum*. Thioredoxin h (B7G7L6, 20%/F2 + 2.10, 10%/F2 + 2.18) was upregulated in both 10% and 20% DMW conditions. H-type thioredoxins have known functions in oxidative stress management (Serrato and Cejudo 2003) and may be involved in the redox regulation of glutathione reductases as seen in *Triticum aestivum* (Gelhay et al. 2003). B7G7L6 shares a 40.4% identity with Trxh1 in *Oryza sativa*, which is induced during abiotic stress, including an elucidated function in managing reactive oxygen species balance in the rice apoplast during salt stress (Zhang and Guo 2012). Increased expression of a Mpv17/PMP-like protein (B7G8P1, 20%/F2 + 1.46) targeting to the peroxisomal membrane provides further evidence of ROS management in the 20% DMW condition. Nitrogen starvation produces oxidative microenvironments in *P. tricornutum* (Rosenwasser et al. 2014), and additional upregulation of a peroxiredoxin (B7GCV6, 10%/F2 + 3.43), an NADP⁺ transhydrogenase (B5Y5A6, 10%/F2 + 1.79), and putative glutathione-s-transferase (B5Y5I2, 10%/F2 + 1.44) in the 10% DMW condition may be required to combat oxidative stress during nitrogen limitation.

Cellular signaling and gene expression

P. tricornutum grown on both 10% and 20% DMW for 5 days exhibited alterations in expression profiles for multiple proteins involved in cellular signaling (Fig. 4f). Compared to the F2 control, hCDK3 (B7FUX2, 10%/F2 + 145) was upregulated in 10% DMW, and CDKA2 (B7GDW6, 20%/F2 -1.41) was downregulated in 20% DMW, suggesting altered regulation of the cell cycle during cultivation on DMW. The upregulations of two calcium-dependent kinases (B7GBS5, 20%/F2 + 1.66, 10%/F2 + 2.86; B7G8H4, 20%/F2 + 2.82, 10%/F2 + 3.33) in both 10% and 20% DMW, along with the upregulation of phospholipase C (B7S3T2, 10%/F2 + 1.78) and copine 1 (B7FSV2, 10%/F2 + 1.30) in 10% DMW, suggest that calcium signaling plays an important role in acclimatizing to culture on this DMW source. Culture on both DMW conditions also downregulated a P-type ATPase (B7GCD8 20%/F2 -1.84, 10%/F2 -1.54) identified as a calcium ion carrier, providing further evidence of modulated calcium signaling pathways. It must be noted, however, that *P. tricornutum* utilizes calcium signaling as part of its iron limitation response (Allen et al. 2008; Falcioratore et al. 2000), and given the iron-limited composition of the DMW used in this study, further studies must be performed to confirm and elucidate the importance of calcium-based signaling during acclimatization to culture on DMW sources.

Three proteins with roles that function as molecular chaperones were downregulated in the 20% DMW condition, including a DnaJ (hsp40) homolog (B7G4P7, 20%/F2 -4.17), an hsp90 homolog (B7GES3, 20%/F2 -4.52), and the molecular chaperone cdc37 (B7S3L1, 20%/F2 -1.60). These proteins are components within a multi-protein complex that function to chaperone various targets, including protein kinases (Arlander et al. 2006). The functional consequence of their downregulation in the 20% DMW condition is unknown but signify further evidence of alterations in signal transduction.

Phaeodactylum tricornutum grown on DMW exhibited differences in proteins involved in transcriptional regulation when compared to culture on synthetic medium. Two transcription factors, one identified as containing an HMG box domain (B7FQD6, 20%/F2, -4.25) and another identified Myb1R2 (B7G7Q5, 20%/F2 -2.32) (Rayko et al. 2010), were downregulated in 20% DMW. An additional protein identified as splicing factor 3B subunit 3 (B7FP73, 20%/F2 -1.97), which functions in pre-mRNA splicing, was also downregulated in the 20% DMW. The CO₂ responsive bZIP11 (B7G9T9, 10%/F2 + 1.34) transcription factor was upregulated in 10% DMW, while CSF4 (B7G5B5, 20%/F2 + 2.62, 10%/F2 + 2.77), a DNA-binding protein containing a cold shock domain, was upregulated in both 10% and 20% DMW. Although this study cannot clearly implicate the functional consequences that result from altering transcriptional regulation and cellular signaling pathways, it may contribute

to the proteomic re-organization observed in *P. tricornutum* in response to DMW cultivation.

Membrane transport and trafficking

Proteomic analysis after 5 days of growth on DMW suggests that *P. tricornutum* responds to DMW conditions by controlling small molecule and protein transport throughout various cellular compartments. Upregulation of small GTPases belonging to the Ras superfamily of proteins were observed in both 10% and 20% conditions, including proteins identified as RabX1 (B7S3S7, 20%/F2 + 1.92, 10%/F2 + 2.29) and Arf1 (B7FR50, 20%/F2 + 3.97, 10%/F2 + 4.32), with the Rab GTPase Sec4 (B7G9L9, 10%/F2 + 1.79) observed upregulated in the 10% DMW condition only (Supplementary Fig. 2). Both RabX1, which shares a 78.9% sequence identity with the Ras-related protein Rab-8A in *Fragilariopsis cylindrus*, and Arf1 are involved in endocytosis. Additional support of increased endocytic pathways was observed in the 10% DMW condition, where upregulation of adaptor protein complex components AP1/2beta (B7S4C6, 10%/F2 + 1.23) and AP2M1 (B7FSB1, 10%/F2 + 1.48) was observed (Supplementary Fig. 3). The increased expression of a V-type H^+ transporting ATPase (B7G360, 10%/F2 + 1.61) in 10% DMW suggests an increased role for phagocytosis in this condition and, taken together, could provide *P. tricornutum* grown on the DMW an additional scavenging mechanism to acquire limiting nutrients such as iron and nitrogen from the outside environment.

Protein trafficking was generally upregulated during DMW culture. The nuclear transport factor NFT2 (B7G8V7, 20%/F2 + 1.57, 10%/F2 + 2.21) was upregulated in both conditions, and the Ran-GTPase importin-7 (B7FVE6, 10%/F2 + 1.21) was upregulated in 10% DMW. Nuclear export by exportin-1 (B7GEG3, 20%/F2 -1.68) was downregulated in 20% DMW. The alpha subunit of sec61 (B7GD39, 20%/F2 + 1.79, 10%/F2 + 1.35), sec31 (B7G255, 20%/F2 + 1.63, 10%/F2 + 2.20), and a protein identified as part of the SRP-independent protein family (B7FUG7, 20%/F2 + 1.44, 10%/F2 + 1.57) were upregulated in 10% and 20% DMW conditions, suggesting an increase in protein translocation through the endoplasmic reticulum (Supplementary Fig. 2), and provide support for the importance of controlling protein synthesis during DMW culturing.

Cultivation on DMW increased the expression of a MFS superfamily transport protein (B7FTZ4, 20%/F2 + 1.46, 10%/F2 + 1.60) that putatively functions in the transport of molybdate ions across membranes. In its biologically active form, molybdenum acts as a cofactor in specific enzymes, including purine catabolism such as xanthine dehydrogenase (B7GAV1, 10%/F2 + 1.28) and nitrogen assimilation nitrate reductase (B7G997, 10%/F2 + 1.35), both of which were upregulated in the 10% DMW condition. Two additional

putative phosphate transporters (B7GBF0, 20%/F2 -3.90 and B7G4H1, 20%/F2 -2.48) were also downregulated in 20% DMW.

Lipid metabolism

Phaeodactylum tricornutum cultivated on either 10% or 20% DMW upregulated an acyl carrier protein (ACP) (B7FRX6, 20%/F2 + 2.50, 10%/F2 + 2.46) and downregulated a stearoyl-ACP desaturase (B7FQK1, 20%/F2 -2.01, 10%/F2 -1.92). ACPs perform important functions as cofactors in fatty acid biosynthesis, and overexpressions of specific ACP isoforms have been demonstrated to alter FA composition (Brannen et al. 2001) and modulate salt stress tolerance in *Arabidopsis* (Huang et al. 2017). Stearoyl-ACP desaturase requires iron as a cofactor, and iron limitation in this medium source may explain the observed downregulation. A decrease in FA desaturase is expected to alter the fatty acid profile towards increased saturation, and in *C. reinhardtii*, iron starvation downregulates expression of multiple FA desaturase genes, increasing the saturated to unsaturated FA ratio while altering the FA composition of membrane lipids (Urzica et al. 2013). Interestingly, a recent study where proteomic analysis was performed on *P. tricornutum* grown on iron-limited or iron-starved F2 medium did not cause downregulation of FA desaturases (Zhao et al. 2018), highlighting microalgae species-specific differences in responding to iron limitation. Indeed, the FA composition of *P. tricornutum* cultured on DMW did not increase FA saturation when compared to culture on F2, and FA unsaturation actually increased in all DMW dilution used besides the nitrogen-limited 10% DMW condition (Table 2).

Conclusions

This study demonstrates the utility of dairy manure wastewater as a media source for *P. tricornutum* cultivation. The fatty acid composition, biomass yields, and lipid yields vary based on the dilution of DMW in seawater (from 10 to 60%), which correlates to starting nitrogen content. Alterations in the proteome identified across cultivation conditions demonstrate that *P. tricornutum* responds to DMW cultivation by altering multiple biological pathways. This study demonstrates that proteomic analysis can contribute to wastewater characterization, e.g., to identify the low iron content of DMW. The demonstration of successful cultivation and significant lipid accumulation of *P. tricornutum* on DMW supports the viability of using low-cost waste nutrients for microalgae biomass and biofuel production, as well as the partial remediation of wastewaters during microalgae cultivation. Overall, this study contributes to greater insight of proteome reorganization of microalgae during wastewater cultivation.

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Declarations

Conflict of interest The authors declare no competing interests.

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