1 Characterization of *Picochlorum* sp. use of wastewater generated from hydrothermal
2 liquefaction as a nitrogen source

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15 ABSTRACT:

16 *Picochlorum* sp. strain *SENEW3* is a halotolerant green alga with high growth rates and
17 the ability to utilize organic nitrogen sources including wastewater generated from
18 hydrothermal liquefaction (HTL-WW). *Picochlorum* acclimates to the presence of HTL-
19 WW. In a photosynthesis/respiration rate test, *Picochlorum* *SENEW3* showed a stress
20 response to HTL-WW additions in a dose-dependent manner while cells pre-grown with
21 HTL-WW had a greatly reduced response to additions. A quantitative proteomics tool,

iTRAQ, was applied to assess *Picochlorum* global proteome changes in response to HTL-WW as a nitrogen source. From this approach, a total of 350 different proteins were identified across 2 biological replicates with significant up-regulated or down-regulated (average ratio more than 1.2 or less than 0.8, at least one p-value <0.05). Protease and oxidative stress enzymes were notably up-regulated. An aminopeptidase enzyme assay showed that, compared to controls, cells grown with 0.1% (vol) HTL-WW had 2.1-fold higher protease activity. An ascorbate peroxidase assay showed an 8.6-fold increase in exponential cells grown with 0.1% HTL-WW compared to controls. This study provides insights into the development of microalgae for algal biofuel production using HTL wastewater recycling[1].

Keywords: Algal biofuels, Hydrothermal liquefaction, Nutrient recycling, Proteomics

1. Introduction

One of the biggest challenges facing the large-scale utilization of algae-based biofuels is to identify sources for the essential nutrients (mainly nitrogen and phosphorus) required for growth. The projected nutrient demands scale in proportion to the algal biomass production volume, leading to increasingly severe nutrient demand challenges at the higher target oil production levels [1]. Nutrients also have been reported as one of the major operational costs for algal biomass production. Based on a reconciled model from life cycle analysis (LCA) and techno-economic analysis (TEA), nutrients account for 10% of gross operation costs [2,3]. Beyond the operational cost, the LCA study shows that nutrients from fertilizer have an environmental cost, which is as high as 3.2 kilogram carbon dioxide per kilogram ammonia nitrogen [2]. Therefore, it would be valuable to pursue options for algal production that enable the effective capture and reuse of nutrients from the production cycle to improve overall life cycle performance [4–6].

Hydrothermal liquefaction (HTL) is a process that directly liquefies wet biomass to crude oil in the presence of water. It creates short-chain petroleum hydrocarbons as well as an aqueous phase and gaseous products. The petroleum hydrocarbons extractable by a non-polar solvent are referred to as crude oil. The aqueous phase, referred as HTL wastewater (HTL-WW) in this study, is rich in organic nitrogen and other nutrients. This would ideally be recycled to grow more algal biomass instead of being discharged, which might cause adverse impacts to the environment.

However, the potential of HTL-WW for nutrient recycling has not been well studied. Jena et al. showed the feasibility of recycling HTL-WW by growing *Chlorella minutissima* with diluted HTL-WW from *Spirulina* in deionized water [7]. Biller et al. demonstrated a closed loop system using recovered HTL-WW from the HTL process for algal growth of eukaryotic microalgae (*Chlorella vulgaris*, *Scenedesmus dimorphus*) and two cyanobacteria (*Spirulina platensis* and *Chlorogloeopsis fritschii*) [8]. It was also found that *Chlorella vulgaris* grown in HTL-WW as the only nutrient source had higher biomass and fatty acid methyl ester yields compared to regular medium [9]. In a recent study, growth reduction was observed when *Desmodesmus* sp. was grown with HTL-WW, which was due to insufficient macro- or micro- nutrients other than N and P [10].

Picochlorum SENEW3 (SE3) was isolated from a small permanent pond in San Elijo Lagoon system in San Diego, California. The pond is subject to large seasonal fluctuations in salinity (1.7 -108.3‰) via evaporation, precipitation, and tidal influx of seawater. *Picochlorum* is a slightly oval, nonflagellated green alga with an approximate mean size of 2 µm. The 18S rRNA sequence of this isolate showed very high percentage (99%) identity with a published 18S rDNA sequence of *Picochlorum* sp., which is classified as belonging to the Domain Eukarya, Kingdom Protista, Division Chlorophyta and Class Trebouxiophyceae [11,12]. As one of the smallest and most gene dense eukaryotic algae, *Picochlorum* SE3 encodes 7,367 genes with 13.3 Mbp genome [13]. It has high growth rates ($>0.5 \text{ day}^{-1}$) over broad temperature and salinity ranges as well as high oil contents [14]. It also shows ability to utilize organic nitrogen sources (manuscript in preparation). For all these reasons, *Picochlorum* SE3 may be a promising

candidate for commercial algal biomass applications and an ideal model strain for genome and proteome studies.

Quantitative proteomic analyses provide an opportunity to enable large scale understanding of how *Picochlorum* SE3 adjusts its proteome and overall metabolism to HTL-WW as a nitrogen source. In order to confirm proteomic data and better understand the responses of *Picochlorum* SE3, enzyme assays were also performed. These data provide insights into finding, acclimating, or engineering algae to be part of an algal biomass recycling system.

2. Methods and Material

2.1 Microalgal cultivation

One mL previously grown *Picochlorum* SE3 was inoculated in 1 L sterile f/2 growth medium or nitrate deficient f/2 medium containing 0.1% (vol) HTL-WW (Table 1). When cells under both treatments reached stationary phase, they were transferred to “bag” photobioreactors inside of a greenhouse with natural light. These photobioreactors were made using 16” or 10” 10 mil low density polyethylene (LDPE) tubing (Landsberg, Pasadena, CA) hung from pallet racks and filled with sterile-filtered 50L of f/2 media or nitrate deficient f/2 medium with 0.1% (vol) HTL-WW. 3/8” HDPE tubing with 4 drilled holes at the bend were inserted into each bag and used to bubble air plumbed in from a blower fan for aeration and mixing. Bubbling rate was adjusted for each bag using a ball valve. Environmental controls in the greenhouse attempted to maintain a temperature of 22 ± 2 °C. Both treatment conditions were duplicates.

Table 1. Composition of HTL-WW sample used in this study

Components	Concentration (ppm)
Nitrogen	
Total Nitrogen	11936
Nitrite	193 (4 mM)
Nitrate	75 (1 mM)
Ammonium	8966 (498 mM)
Total Kjeldahl Nitrogen	4887
Carbon	
Total Carbon	39220
Total Organic Carbon	34920
Inorganic Carbon	4292
Phosphate and other nutrients	
Phosphate	3072 (32 mM)
Fluoride	1994 (105 mM)
Chloride	1109 (31 mM)
Sulfate	545 (6 mM)
Bromide	43 (1 mM)
Sodium	3507 (152 mM)
Potassium	3204 (82 mM)

Pond cultures were grown in 320 gallon (1200L) round stock tanks (Den Hartog Industries, Inc., Hospers, IA) with a final volume of 800L media at a depth of approximately 40 cm. Ponds were inoculated with greenhouse bag cultures. Ponds were mixed using an air stone and airlifters constructed with PVC pipe connected to a blower fan. Nutrients were added when the nitrate and phosphate concentration were below 5ppm. Pond cultures were harvested periodically using a continuous flow centrifuge at 3000RPM. Chlorophyll was recorded daily using an Aquafluor portable fluorometer (Turner Designs, Sunnyvale, CA). Optical density (OD) readings were taken on a Thermo Spectronic 20 at 750 nm (Thermo Fischer Scientific, Waltham, MA). Biomass was measured before and after harvests based on the protocol recommended by the Algal Biomass Organization [15].

2.2. Photosynthetic and respiration measurements

Photosynthetic and respiration responses to various HTL-WW concentrations were determined using a Clark oxygen electrode (Hansatech instruments LTD, UK) as described in Garbayo et al.[16]. Briefly, 2mL of exponential phase *Picochlorum* SE3 culture were placed in the electrode chamber and treated with several light/dark cycles. At the beginning of each cycle, HTL-WW was spiked into the chamber to the final volume concentrations of 0.1%, 0.2%, 0.5%, 1%, 2% and 5%. The reaction chamber was illuminated with 100 $\mu\text{E}/\text{m}^2$ of LED light during the light cycles and maintained at 22 °C. Respiration was determined for the same sample by darkening the chamber containing the cells. Cell number was determined in a hemacytometer (Hausser Scientific Co., Horsham, PA). The photosynthetic and respiration measurements were obtained by normalizing the oxygen production or consumption rates in light or dark cycles to cell concentrations.

2.3. iTRAQ protein preparation and analysis

Cells for iTRAQ analysis were sampled from photobioreactors. 1 liter exponential phase cells were collected when chlorophyll levels reached approximately 30 RFU (Stationary phase was 120 RFU). Cells were washed and re-suspended in approximately 4 mL of 100 mM MET. The concentrated cells were broken in a French Pressure Cell Press (SLM Instrument, Inc). Total proteins of extract sample slurry were measured using Pierce BCA Protein Assay Kit (Thermo scientific, Rockford, IL) and stored at -80 °C for future analysis.

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141 Protein samples were sent to the Department of Chemistry and Biochemistry, UCSD for
142 further analysis. Briefly, protein samples diluted in TNE buffer (50 mM Tris pH 8.0, 100
143 mM NaCl, 1 mM EDTA) were boiled with RapiGest SF reagent (final concentration
144 0.1%, Waters Corp.) for 5 min. TCEP (Tris (2-carboxyethyl) phosphine) was added to 1
145 mM (final concentration) and incubated at 37°C for 30 min. Subsequently, the samples
146 were carboxymethylated with 0.5 mg/ml of iodoacetamide for 30 min at 37°C followed
147 by neutralization with 2 mM TCEP (final concentration). Proteins samples prepared as
148 above were digested with trypsin (trypsin:protein ratio - 1:50) overnight at 37°C.
149 RapiGest was degraded and removed by treating the samples with 250 mM HCl at 37°C
150 for 1 h followed by centrifugation at 14000 rpm for 30 min at 4 °C. The soluble fraction
151 was then added to a new tube and the peptides were extracted and desalted using Aspire
152 RP30 desalting columns (Thermo Scientific).

153

154 The four trypsinized samples were then pooled and fractionated using high pH reverse
155 phase chromatography (HPRP-Xterra C18 reverse phase, 4.6 mmx10 mm 5µ particle
156 (Waters)). The chromatography conditions were as follows: the column was heated to
157 37°C and a linear gradient from 5-35% B (Buffer A-20 mM ammonium formate pH 10
158 aqueous, Buffer B-20 mM ammonium formate pH 10 in 80% Acetonitrile water) was
159 applied for 80 minutes at 0.5 ml/min flow rate. A total of 48 fractions of 0.5ml volume
160 were collected for LC-MSMS analysis. Each of these fractions were analyzed by high
161 pressure liquid chromatography (HPLC) coupled with tandem mass spectroscopy (LC-
162 MS/MS) using nano-spray ionization. The nanospray ionization experiments were

performed using a QSTAR-Elite hybrid mass spectrometer (ABSCIEX) interfaced with nano-scale reversed-phase HPLC (Tempo) using a 10 cm-180 micron ID glass capillary packed with 5- μ m C18 ZorbaxTM beads (Agilent Technologies, Santa Clara, CA). Peptides were eluted from the C18 column into the mass spectrometer using a linear gradient (5–30%) of Acetonitrile at a flowrate of 550 μ l/min for 100minutes. The buffers used to create the ACN gradient were: Buffer A (98% H₂O, 2% ACN, 0.2% formic acid, and 0.005% TFA) and Buffer B (100% ACN, 0.2% formic acid, and 0.005% TFA). MS/MS data were acquired in a data-dependent manner in which the MS1 data was acquired at m/z of 400 to 1800 Da and the MS/MS data was acquired from m/z of 50 to 2,000 Da [17–20].

2.4. Protein statistical analyses

ProteinPilot software v.4.0 (Applied Biosystems, MDS-Sciex) was used to identify and quantify proteins with the Paragon search algorithm. The sequence data and the assembled contigs of *Picochlorum* SE3 genome used for protein search can be accessed via NCBI BioProject PRJNA245752 [13]. Ratios between HTL-WW treated and nitrate control replicates were generated using ProteinPilot software. Proteins with less than one unique peptide hit at 95% confidence and less than 1.2 used number (equal to 95% false discover rate) were discarded. The heatmap was generated in R (2.14.0) using the heatmap.2 function of gplots package (<http://cran.r-project.org/web/packages/gplots/index.html>)

2.5. Aminopeptidase enzyme assay

Aminopeptidase activity was measured as described in Martinez and Azam [21] using the hydrolysis rate of L-leucine 7-amino-4-methylcoumarin (leu-AMC; Sigma), which releases the highly fluorescent product 7-amino-4-methylcoumarin (AMC). *Picochlorum* SE3 cultures were treated with kanamycin prior to testing. Fifty microliter of cells were inoculated to 20 mL sterile f/2 medium or f/2 nitrate deficient with 0.1% HTL-WW. Axenic cultures were maintained in Pyrex® flat bottom culture tubes at 21 °C, and exposed to continuous illumination at a light intensity of 30~50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ from 54W T5 fluorescent bulbs. Exponential phase cells were collected, washed twice in fresh medium (f/2 without nitrogen) and incubated with 0.25 M EDTA pH 8.0 and 1.25 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP; Sigma-Aldrich Co., St Louis, Mo, USA) for 20 minutes. The supernatant of the cell culture after incubation was filtered and collected through 0.2 μm filters (Supor® PES membrane, Pall corporation). Cells without EDTA treatment were served as controls. A portion of EDTA-treated cells was washed twice with fresh medium to compare with the unwashed EDTA-treated cells. All cells and supernatants were tested for aminopeptidase activity. 50 mM Leu-AMC, dissolved in ethylene glycol monomethyl ether, was added to the sample to a 100 μM final concentration. Increase in fluorescence with time was measured (excitation, 365 nm; emission, 460 nm) at room temperature in a Molecular Devices Spectra Max M2 spectrophotometer supplied with SoftMax® Pro Data Acquisition & Analysis Software (Molecular Devices LLC, Sunnyvale, CA). Cells were counted in a hemacytometer (Hausser Scientific Co., Horsham, PA).

2.6. Ascorbate peroxidase (AsPOX) assay

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210 *Picochlorum* SE3 was grown in photobioreactors as described in section above. Both
211 exponential and stationary phase cells were collected, washed with fresh medium and
212 stored at -80 °C. Cells was thawed on ice and broken by French Pressure Cell Press
213 (SLM Instrument, Inc) three times. The supernatant as crude enzyme extract was
214 obtained by centrifuging at 18000 rpm for 20 min at 4 °C and analyzed immediately
215 using AsPOX assay modified from Venisse et al [22]. Briefly, 2 µl crude enzyme extract
216 was added to 200µl reaction mix composed of 0.2 M Tris-HCl buffer at pH 7.8, 0.25 mM
217 ascorbic acid and 0.5 mM H₂O₂. The decrease of absorbance at 290 nm was immediately
218 recorded using a fluorescence spectrometer (SpectraMax M2, Molecular Devices). The
219 linear decrease rates indicated the AsPOX activity.

220

221 2.7 Photobleaching of raffinose

222 Sterile-filtered HTL-WW was added to sterile seawater and was incubated under
223 fluorescent lights or dark conditions. Fluorescence in the red (chlorophyll channel) on a
224 Turner designs model AU10 fluorometer was measured regularly for one week. The
225 fluorescence of seawater-raffinose samples in the light declined, while samples in the
226 dark did not, indicating that raffinose was photo-bleaching (data not shown).

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230 3. Results and Discussion

231 3.1. *Algal pond cultivation*

Figure 1 shows the growth of *Picochlorum* SE3 in outdoor algal miniponds from May to July in 2013 using initial HTL-WW levels of 0.1% (vol). The pond was inoculated and *Picochlorum* SE3 grew exponentially and reached stationary phase when the nutrients were depleted. Additional HTL-WW was added and the growth resumed. Harvesting brought down the cell concentration but additional HTL-WW supported pond re-growth. After the nutrients were completely exhausted, the cell concentration eventually dropped to nearly zero. The oil contents of harvested cells on June 7 and June 24 ranged from 38.17~39.47%. The nitrogen recovery rates were 40.6% and 82.6% respectively.

Figure 1. Growth of *Picochlorum* SE3 in algal ponds on seawater and 0.1% (vol) HTL-WW as the nutrient source. HTL-WW was added and cells were harvested periodically. Symbols ○ and ● indicate 0.02% and 0.04% (vol) HTL-WW were added, respectively.

3.2. Photosynthetic oxygen measurements

The additions of HTL-WW had significant effects on the photosynthesis and respiration rate of *Picochlorum* SE3 in the lab studies. Figure 2 shows that HTL-WW additions

inhibited both the photosynthesis and respiration of *Picochlorum* SE3 in a dose-dependent manner. Photosynthesis of nitrate-grown cells was largely gone at 1% HTL-WW, but cells pre-grown on 0.1% HTL-WW were acclimated and continued photosynthesis at 2% HTL-WW. Similarly, the respiration rate of nitrate-grown cells dropped to zero at 2% HTL-WW while HTL-WW grown cells were still able to maintain respiration. The inhibition effect of HTL-WW on *Picochlorum* SE3 may be from the toxicity of its components such as phenol, fatty acid, solvent residues or metals from reactor corrosion during the HTL processes [8].

Photosynthesis and respiration have been used for decades to assess the metabolic rate of organisms. Generally, Cells with high photosynthesis and respiration rates tend to have high growth rate and accumulate more biomass [23,24] Photosynthesis and respiration rates also help to describe acute responses of various phytoplankton to stress factors such as temperature, irradiance, etc [25,26]. However, they only represent the summation of many processes, each of which may react differently to the applied stress factors.

Figure 2. Cellular respiration and photosynthesis rates per cell of *Picochlorum* SE3 grown on f/2 medium (triangles in dash lines) and on f/2 nitrate deficient medium with 0.1% HTL-WW (squares in solid lines). Respiration rates (open symbols) were measured in dark chamber while photosynthesis rates (solid symbols) were in $100 \mu\text{m}^2 \text{s}^{-1}$ light intensity.

3.3 Experimental design and statistical interpretations

Experiments were performed to compare proteome differences of *Picochlorum* SE3 when using HTL-WW as the only nitrogen sources for growth. The biological replicates of control protein samples are labeled with N1 and N2 while HTL-WW treated cell protein samples were labeled with HTL1 and HTL2.

The false discovery rate (FDR) analysis was first performed to reduce the risk of false positive identification. 2836 proteins were identified at an error rate of 5% for local FDR,

which corresponds to a value of 1.2 for unused peptides in the ProteinPilot program. Considering the whole genome of *Picochlorum* SE3 contains 7367 proteins, this result suggests that expression changes were quantified for 38% of theoretical ORFs.

The list of identified proteins was further reduced by the constraint of more than 1 unique peptide identified. For comparison purposes, four ratios, HTL1/N1, HTL2/N1, HTL1/N2 and HTL2/N2, were used. Proteins significantly up-regulated or down-regulated (average ratio more than 1.2 or less than 0.8, at least one p-value <0.05) were selected. In the end, 350 proteins were obtained (Set A). Putative annotations of these quantified proteins, top database hit, and other attributes are added based on their contig of origin. The list of quantified proteins with quantitative and attribute data is provided in the Supplement 1 in Supporting Information.

To further narrow the search for regulated proteins, a stricter criterion was used: the averages of four ratios of each quantified protein were either more than 1.2 or less than 0.8 with all p-value<0.05. 56 proteins were found to be significantly changed based on such criteria (Set B). Of these, 31 proteins were up-regulated and 25 were down-regulated compared to control samples. The list of quantified proteins with quantitative and attribute data is provided in the Supplement 2 in Supporting Information. Figure 3 shows the heatmap and the hierarchical clustering of 56 proteins. Major changes were observed in proteins participating in energy production, photosynthesis, amino acid metabolism, as well as cell envelope biogenesis. This result demonstrates that iTRAQ methodology can systematically quantify the proteome responses in *Picochlorum* on a

301 global scale. The use of two independent replicates and statistics tools allows confidence
 302 in all observable changes. As shown in Figure 3, two biological replicates under the same
 303 condition show very high similarity. The observed differential regulation was generally
 304 consistent in all biological replicates.

305 Figure 3. The heatmap and the hierarchical clustering of 56 proteins up- or down-
 306 regulated when exponential *Picochlorum* SE3 used HTL-WW or nitrate as the only
 307 nitrogen source. Up-regulated and down-regulated proteins are indicated in shades of red
 308 and green, respectively. Color intensity is described by the bar in the top left corner. Six
 309 proteins with unknown function are not shown. They are included in Supplement 5.

310 * Ascorbate peroxidase, see discussion

311 ** Protease, see discussion

312

313 Table 2. Top ten up-regulated and down-regulated proteins for Set A (375 proteins) and

314 B (56 proteins)*

	Set A		Set B	
	Protein	avg. ratio	Protein	avg. ratio
up-regulated	ef-hand domain-containing thioredoxin	17.33	ef-hand domain-containing thioredoxin	17.33±12
	acetamidase formamidase	17.22	glyceraldehyde-3-phosphate type i	12.66±4.4
	glyceraldehyde-3-phosphate type i	12.66	aldehyde-alcohol dehydrogenase	12.26±3.7
	aldehyde-alcohol dehydrogenase	12.26	light-harvesting chlorophyll a b-binding protein	10.03±4.1
	transcription factor gata-4	10.84	glutamine synthetase	9.54±1.8
	light-harvesting chlorophyll a b-binding protein	10.03	alanine-anticapsin ligase	8.53±5.0
	glutamine synthetase	9.54	light-harvesting protein of photosystem i	7.36±2.8
	eukaryotic translation initiation factor 4e	9.37	kynurenine 3-monooxygenase and related flavoprotein monooxygenases	7.19±4.1
	chlorophyll a b-binding protein	9.07	l-ascorbate peroxidase	6.98±2.6
	alanine-anticapsin ligase	8.53	dehydroascorbate reductase	6.30±4.2
down-regulated	cf0 atp synthase subunit ii precursor	0.059	magnesium chelatase subunit of protochlorophyllide reductase	0.16
	50s ribosomal protein l28	0.057	30s ribosomal protein s4	0.13
	proliferation-associated protein 2g4-like	0.054	60s ribosomal protein l11	0.13
	ribosomal protein l30	0.053	ribosomal protein s20	0.085
			40s ribosomal protein s3	0.080

60s ribosomal protein l28-1	0.052	40s ribosomal protein s15	0.067
60s ribosomal protein l24	0.047	60s ribosomal protein cf0 atp synthase subunit ii precursor	0.060
60s ribosomal protein	0.045	proliferation-associated protein 2g4-like	0.059
60s ribosomal protein l2	0.031		0.054
50s ribosomal protein l34	0.030	50s ribosomal protein l34	0.030

* Proteins with unknown function are not shown in this Table (6 of Set A and 3 of Set B).

They are included in Supplement 5.

Table 2 summaries the top 10 proteins most up-regulated or down-regulated for both Set A and Set B of quantified proteins. Ef-hand domain-containing thioredoxin is the first in both sets with an upregulation under HTL-WW of 17.33. However, since its blast mean similarity is 52% and its expression ratio has high standard deviation, we did not take this protein into further consideration. Alanine-anticapsin ligase, kynurenine 3-monooxygenase and dehydroascorbate reductase similarly had somewhat weak annotations. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) type I, is involved in glucose metabolic process as well as oxidation-reduction. The increase of GADPH may be related to the high content of organic carbon (Table 1) and the toxicity of HTL-WW. Glutamine synthetase (GS), a key enzyme to incorporate ammonia into the carbon skeleton, is increased by 9-fold in HTL-WW cultures compared to nitrate grown cells. Hirel et al.[27], reported that availability of ammonium ions enhanced GS expression in plants. This may explain that the increase in protein expression of GS may be related to the high portion of ammonia in HTL-WW. Aldehyde-alcohol dehydrogenase (ADHE) is responsible for the oxidation of aldehydes to carboxylic acid. Since ADHE sequences were only available for the enzyme from the cytosol of anaerobic eukaryote [28], the expression of ADHE under aerobic conditions is unexpected. Light-harvesting

chlorophyll a b-binding protein can be increased with excessive light. However, it may also be induced in the dark by acetate, which stimulates cellular growth [29]. Since both conditions in the study have the same light intensity, acetate from the HLT-WW may be the reason for the increase.

Compared with the up-regulated proteins, down-regulated proteins were less specific. They are mostly proteins of unknown function or involved in cell growth.

We found that l-ascorbate peroxidase (AsPOX) was significantly increased (6.98 times higher) when cells used HTL-WW rather than nitrate as their nitrogen source. Meanwhile, several predicted antioxidant enzymes were up-regulated as well such, as superoxide dismutase (SOD) with an increase rate of 3.9 (Supplement 4 in Supporting Information). These responses indicate a state of oxidative stress. This may be toxic materials in HTL-WW or due to the photobleaching of HTL-WW noted in methods as this could produce superoxide, but this was not measured. During the experiment, factors such as salinity, pH and temperature were closely monitored and therefore excluded.

Although not shown in Table 2 of top ten changes, several peptidase related enzymes are found in both Set A and Set B, most of which are all up-regulated (Supplement 3 in Supporting Information). High hydrolysis rates of peptides and other organic nitrogen have been reported on eukaryotic picoplankton [26,30]. Our previous studies found that *Picochlorum* SE3 was able to utilize organic nitrogen in forms of glutamic acid, casein or HTL-WW as the only nitrogen source (manuscript in preparation). Therefore,

aminopeptidase enzyme assays were performed to examine cellular aminopeptidase enzyme activity and some of its characteristics.

3.4 Aminopeptidase enzyme assay

The aminopeptidase activities of exponential *Picochlorum* SE3 growth on f/2 medium or f/2 nitrate deficient with 0.1% HTL-WW were measured and normalized to cell concentrations (Figure 4). Sterilized f/2 and EDTA solution were used as negative controls. Supernatants filtered from control cells and EDTA-treated cells were tested as well. For control cells without EDTA treatment, *Picochlorum* SE3 grown with HTL-WW had significant higher aminopeptidase activity compared to cells grown with nitrate (Figure 4). This is consistent with the proteomic results which showed multiple peptidases were up-regulated. None of the negative controls or supernatants from control cells had significant aminopeptidase activity (data not shown), which suggests that aminopeptidase activity was only present with cells and this enzyme is not secreted into the medium. However, supernatants from EDTA-treated cells had low but statistically significant activities. This suggests that EDTA may be able to wash off aminopeptidase enzyme from cells. However, from the comparison of control cells and EDTA-treated cells, it seems that EDTA inhibited the aminopeptidase activities (Figure 4). This finding is similar to a previous study that found EDTA inhibited the protease of marine cyanobacteria in a dose-dependent manner [31]. We were able to remove the EDTA inhibition from nitrate grown cells by washing cells twice with fresh medium. As shown in Figure 4, aminopeptidase activity of these cells was recovered, which indicates that the

EDTA inhibition was reversible. The unrecovered activity from HTL-WW grown cells may due to the EDTA removal of aminopeptidase activity or an irreversible inactivation of a specific peptidase. Leu-AMC is not thought to rapidly cross cell membranes, at least in some microbes [21]. The simplest explanation of our results is that *Picochlorum* cells have a surface located aminopeptidase that is upregulated under HTL-WW, but proof of this will require biochemical studies beyond the scope of this paper.

Figure 4. Aminopeptidase activity per cell of exponential *Picochlorum* SE3 grown on f/2 medium or f/2 nitrate deficient with 0.1% HTL-WW. * indicates t-test p-val<0.05.

Plasma membrane-associated aminopeptidase has been reported for *Chlamydomonas reinhardtii* [32], *Gonyaulax polyedra* [33] and *Aureococcus anophagefferens* [30]. Although the comparison of their aminopeptidase activities is difficult due to different tested substrates, these strains were all able to scavenge nitrogen from organic nitrogen pool. The increased expression of aminopeptidase in *Picochlorum* SE3 grown with HTL-WW may be caused by the nitrogen composition of HTL-WW. Nitrogen starvation was

also found to lead to an increase in protease activity in chlorophyte *Dunaliella tertiolecta* and diatom *Thalassiosira weissflogii* [34]. In a transcriptome study on *Nannochloropsis gaditana*, aminopeptidase increased in response to nitrogen deprivation alone with other genes responsible for protein degradation and nitrogen shuffling [35].

3.5 Ascorbate peroxidase assay

Ascorbate peroxidase (AsPOX) is an integral component of the ascorbate-glutathione cycle which detoxifies peroxides using ascorbate as a reductant [36]. The enzyme assay uses the oxidation of ascorbic acid to measure the activity of AsPOX. To obtain the linear decreasing of ascorbic acid and avoid its oxidation by the air, we optimized the crude enzyme:ascorbic acid ratio and the recording time (not shown). Figure 5 shows the AsPOX activities in vitro of *Picochlorum* SE3 grown in nitrate or HTL-WW. In exponential phase, HTL-WW grown cells expressed 8.5-fold higher (p-value<0.1) AsPOX activity than nitrate grown cells. However, when cells reached their stationary phase, the difference between HTL-WW and Nitrate decreased to 1.5 fold. The AsPOX level of stationary cells grown in nitrate increased 2-fold (p-value<0.1) compared to exponential cells. One of the possibilities is that the stationary cells may start to experience nutrient limitation and oxidative stress.

AsPOX and other antioxidant enzymes are usually found in plants to be co-regulated and their activities increase in response to stress such as excess light [37], drought [38], heat [39] and pathogens [22]. The possible stress factors from HTL-WW may include ammonia, organic components such as fatty acids or solvent residues, metals from reactor corrosion during HTL processes. Although the toxicity of ammonia is complicated, it has

been reported to generate oxidative stress and elevate catalase, guaiacol peroxidase and glutathione peroxidase level in aquatic plants [40]. Organic components of HTL-WW may be another source of toxicity. Petroleum pollutants, pesticides and herbicides have been proven to induce oxidative stress in green algae, dinoflagellates and cyanobacteria [41–43]. Although we are not clear about the exact organic components of HTL-WW due to limited information, we suspected that they may have similar effects. In addition, the photobleaching of HTL-WW mentioned above may generate free radicals that cause oxidative stress.

4. Conclusion

In summary, our results show that *Picochlorum* can growth on HTL-WW in the lab and in outdoor mini-ponds. Induction of peptidases may help it use HTL-WW if there are residual peptides in the waste and the induction of antioxidant systems may help protect it from the toxicity of HTL-WW. These may help explain its robust growth on HTL-WW compared to other algae. It might be possible to engineer these enzyme systems into other algae that might have other useful properties (lipid profiles, etc) so that they can be grown on HTL-WW as well in the future as part of sustainable algal biomass production.

Figure 5. Ascorbate peroxidase activity of *Picochlorum* SE3 grown on f/2 medium (nitrate) or f/2 with 0.1% HTL-WW as a nitrogen source.

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