COMPARATIVE PHYSIOLOGY OF TWO POIKILOHALINE *PICOCHLORUM* STRAINS

By

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Abstract: The *Picochlorum* genus was first circumscribed in 2004, and has since had numerous species and strains added and characterized. This study uses batch and semicontinuous chemostat culture modes to investigate the physiology of *Picochlorum oklahomense* and *P*. sp. SENEW3, in relation to salinity acclimation and shock. Though these two strains are closely related with >99% rRNA identity, they show marked differences in growth rate, nitrogen uptake, and lipid content in response to salinity stress. Additionally, while each strain is shown to accumulate free proline as an organic osmolyte, the timing and magnitude of proline accumulation differs significantly between strains. While both strains utilize proline in a similar manner in salinity shock, *P. oklahomense* relies more heavily on proline in salinity acclimation, indicating that *P*. sp. SENEW3 must have additional salinity tolerance mechanisms. *P. oklahomense* was also shown to have an increased lipid content at high salinity but grew slower than *P. sp. SENEW3*. The results of this physiological characterization support past transcriptomic evidence and help to explain how the environment shapes the physiology and stress response of organisms.

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INTRODUCTION

Background

With the current technology and understanding of algal physiology, microalgae-based biofuels have the potential to reduce the use of fossil fuels with greater land area use efficiency than traditional biofuel crops [1]. However, to date biofuels from algae have not been able to compete with traditional biofuel crops or fossil fuels due to the high cost of large scale cultivation [2]. A recent integrated techno-economic and life-cycle analysis showed that improvements in both the policy environment and technology are necessary to bring down the cost of algae biofuels to compete with fossil fuels [3]. In addition to biofuels, bioproducts from algae such as astaxanthin, β-carotene and the omega-3 fatty acids eicosapentaenoic acid and docosahexaenoic acid are being produced and marketed globally, and bringing down the cost of production would benefit algal bioproducts producers as well as consumers [4]. It is therefore necessary to better understand the basic physiology of this group of organisms in order to make algae bioproducts more competitive with existing commodities.

Nitrogen limitation

Nitrogen limitation is a particularly well studied strategy used to increase lipid accumulation in microalgae. Normally microalgae contain predominantly three kinds of organic substance: protein, carbohydrate and lipid. Under N-limitation, protein synthesis is inhibited and microalgal cell division ceases. However, photosynthesis must continue in the light, and the assimilation pathway of CO_2 is switched to neutral lipid as a storage of energy, increasing the lipid content per microalgal biomass. Numerous studies have shown that microalgae accumulate neutral lipids in response to nitrogen limitation [5-7]. While nitrogen supply is a major factor controlling lipid content, N-limitation has long been known to reduce growth and total biomass accumulation in addition to inducing changes in the type of fatty acids accumulated [8, 9]. In semi-continuous chemostat cultures of *Chlorella sorokiniana* and *Oocystis polymorpha* subjected to successively decreasing nitrogen supply, growth parameters including oxygen evolution and biomass production decreased as cellular nitrogen content decreased from 10% to 4% of dry weight, but no lipid accumulation was observed and the cultures were unsustainable in semi-continuous culture due to low growth rate under low N supply [10]. In batch cultures, lipids accumulated only when cellular nitrogen content dropped to 3% of dry weight, demonstrating the trade-off between growth and lipid accumulation [10]. Similarly, *Neochloris oleabundans* showed higher biomass accumulation in nitrogen replete batch cultures but a higher cellular lipid content in nitrogen deficient conditions [11]. To offset the marked decrease in growth rate observed under nitrogen limitation, two stage systems have been developed and tested that first grow large quantities of biomass under nitrogen replete conditions, then transfer the cultures to nitrogen deficient

media for the lipid accumulation stage [12, 13]. Comparative physiology studies of both marine and freshwater microalgae have shown that the response to nitrogen limitation varies considerably between lineages in terms of photosynthetic efficiency, biomass composition and productivity, and lipid content, highlighting the need for research into the species-specific factors that contribute to the observed differences in nitrogen limitation-induced lipid accumulation between species [14, 15].

Salinity stress

High salt concentrations also place a burden on the growth of algae, even in nutrient replete conditions. In a comparison of both marine and freshwater species of *Picochlorum* and the ambiguously resolved "*Nannochloris*-like" clades, increases in salinity resulted in similar decreasing trends in growth rate for both marine and freshwater strains, but over different optimal salinity ranges [16]. A common strategy for halotolerant microalgae is to produce and accumulate compatible osmolytes, small organic molecules that balance osmotic pressure without interfering with protein function. These can include carbohydrates such as glycerol and sorbitol, amino acids such as glycine and proline, and methylamines such as glycine betaine [17]. These molecules are a part of normal metabolic pathways, but are produced in much higher quantities under hypersaline stress in order to maintain osmotic pressure with the extracellular environment. In addition to producing and accumulating intracellular compatible osmolytes, species of *Chlamydomonas* form multicellular palmelloid aggregates and exude extracellular polysaccharides, while other algal species upregulate membrane transport proteins when exposed to salt stress [18]. Members of the genus *Picochlorum* have been shown to increase intracellular proline content in response to increased salinity

although the exact timing and magnitude of proline accumulation was not investigated [19, 20]. Free proline maintains osmotic balance in response to salinity stress, and also serves to stabilize proteins, scavenge reactive oxygen species, and maintain redox balance [21, 22]. Transcriptomic evidence shows that starch synthesis is upregulated in high salinity, precluding the accumulation of glycerol, which is formed from products of the breakdown of starch, and also that enzymes leading to proline synthesis are up-regulated [19]. This indicates that proline rather than glycerol is the major osmolyte in *P.* sp. SE3.

Stress concepts

It is important to consider four key factors in algal physiological stress: the optimal conditions for the alga, the magnitude of the stress, the duration of the stress, and perhaps most importantly, the physiological state of the alga at the time the stress is applied [23]. When considering optimal conditions, the environment in which the organism evolved plays a large role; freshwater algae may be extremely stressed at 3.5% w/v salinity, but marine algae may experience stress when transferred to 0% salinity. The magnitude and duration of a stressor determine the ability of the organism to acclimate to stressful conditions. The physiological state of the alga at the time when stress is applied is determined by factors including the growth stage, nutrient supply, and how close to optimal conditions the algae is grown. This is important because the physiological state determines how well the alga is able to initiate and maintain the physiological changes necessary to counteract the stress. In a study investigating the timing, magnitude, and initial conditions of salt stress on the marine alga *Dunaliella tertiolecta*, increasing initial salinity of batch cultures above 5.8% NaCl decreased growth but increased lipid content

compared to 2.9% NaCl, while the same salinity increase applied at mid-log phase had less of a negative impact on growth rate while still causing the cells to accumulate a higher percentage of lipids [24]. In batch cultures of the freshwater alga *Scenedesmus obliquus*, a similar trend of decreasing growth rate and increasing lipid content was seen with increasing salinity, but the effect of the timing of the salt stress was not investigated [25].

Organisms of interest

The green microalga *Picochlorum oklahomense* is a broadly halotolerant chlorophyte belonging to the order Chlorellales, class Trebouxiophyceae [20]. Isolated from an ephemeral hypersaline puddle in the Great Salt Plains National Wildlife Refuge (GSP) in Alfalfa County, Oklahoma, this organism has evolved physiological mechanisms that allow it to grow in environments with a high degree of variability in salinity. Salinity of the global oceanic euphotic zone varies narrowly between 3.15-3.7% w/v, while salinity of surface water at the GSP ranges from near-freshwater during floods to >30% in saturated brine pools [20, 26]. Additionally, a study of GSP surface waters showed spatial and seasonal variability in salinity, ranging from 11.8% near West Clay Creek in summer to 0.1275% near the Salt Fork River in winter [27]. This environmental variation selects for organisms living in the GSP that are able to tolerate widely varying salinity, and may have contributed to the divergence of *P. oklahomense* from other closely related taxa. Experimental results suggest that acclimation to high salinity also increases heat tolerance in this species [28]. Preliminary metabolite analysis of *P. oklahomense* grown at 0 and 4% NaCl has shown that the amino acid proline is the main organic osmolyte produced under high salinity, nutrient replete, growth conditions [29].

Picochlorum sp. SE3, obtained from the Bhattacharya lab at Rutgers University, was isolated from a 160 m^2 poikilohaline pond adjacent to the San Elijo Lagoon with occasional seawater inundation n San Diego County, California [30]. This strain grows in media up to 8.76% salinity and has had transcriptional analysis performed under different salinities [19]. However, its growth has not been studied under simultaneous N-limitation and variable salinity, therefore making it a prime candidate for comparison to *P. oklahomense* under these conditions. *P.* sp. SE3 is expected to have a similar general trend, but with some differences due to differential genome organization. Both strains have published genomes that each have significant unique features compared to other published *Picochlorum* genomes, including differential prokaryotic horizontal gene transfer events and the presence of operon-like functional gene clusters in *P.* sp. SE3 [31-34]. Under high salinity conditions, upregulation of nitrogen assimilation enzymes responsible for nitrate transport across the cell membrane, nitrate reduction, nitrite transport, and nitrite reduction, as well as the proline synthesis enzyme pyrroline-5-carboxylate reductase, one hour after hyper-saline shock suggests that *P.* sp. SE3 uses proline as a compatible osmolyte [19]. As a nitrogen containing molecule, accumulation of proline may lead to a higher requirement for nitrogen, thereby exacerbating nitrogen limitation and causing increased lipid accumulation in both strains. However, *P.* sp. SE3 has differential gene expression from *P. oklahomense* in response to high salinity, necessitating the need for physiological characterization [32]. Investigating the physiology of these two organisms will help shed more light on the physiology of genus *Picochlorum* and how this clade has adapted to dynamic salinity growing conditions.

CHAPTER II

METHODS

Organism sources

P. oklahomense has been continually subcultured at Oklahoma State University since its isolation in 1998. It was the source of strains UTEX 2491 and CCMP 2329 except that our cultures have not been cryopreserved. *P.* sp. SE3 was graciously shipped from the lab of Debashish Bhattacharya at Rutgers University but has not yet been submitted to a culture collection.

Culture modes

We employed two culture modes to examine nitrogen and salinity stress responses under different initial physiological states: batch and semicontinuous, all on a 14:10 h L:D cycle. Batch cultures exhibit rapidly decelerating growth as nutrients are depleted, thus constantly changing physiology even without superimposed stress treatments. In contrast, semicontinuous cultured cells maintain a stable specific growth rate and physiological state from day to day, in the absence of experimental perturbation.

Batch cultures. Both strains were acclimated to 1, 4, and 7% w/v NaCl artificial seawater (AS) minus silica media by transferring into successive batch cultures for at least 10 generations, determined by cell count. These salinity acclimated cultures were then inoculated into six replicate flasks to achieve hypo-, iso-, and hypersaline treatments according to Fig. 1. Salinity was verified using a salinity refractometer. Media contained 500 µM $NaNO_3$ and 370 µM KH_2PO_4 in order to impose nitrogen limitation, with an initial cell density of approximately $1x10^6$ cells mL^{-1}. Cultures were grown on an orbital shaker in 250 mL flasks with 100 mL culture volume under 1000 W metal halide lamps with a photon flux of 200 µmol m^{-2} s^{-1} set to a 14:10 hour L:D cycle, with an ambient temperature of 20 ˚C. Daily, predawn 1-mL samples were collected and used for turbidity, cell count, NO_x, and BODIPY measurements. On days 5 and 10, triplicate cultures from each treatment were harvested for proline content assays.

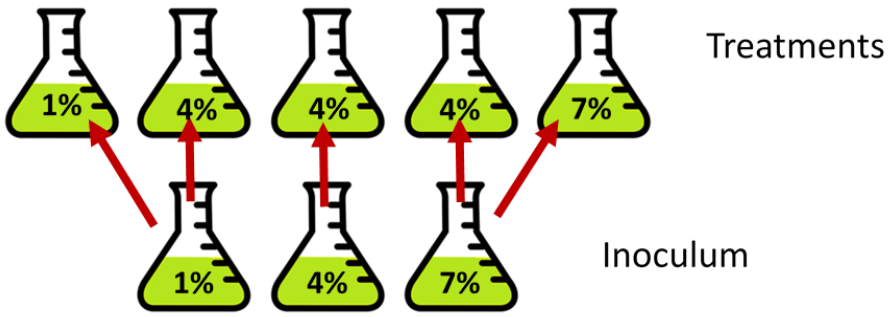

Fig. 1. Batch culture salinity acclimation and shock design. Each strain was acclimated to 1, 4, and 7% salinity for >10 generations before collecting samples at isosaline conditions for each salinity and for hypersaline shock from 1-4% and hyposaline shock from 7-4%.

Semicontinuous chemostat. Both strains were inoculated into six replicates with 200 mL of 1% AS media minus silica, at an initial cell density of $5x10^6$ cells mL^{-1}. To

allow the cultures to equilibrate to culture conditions, 100 mL of culture was decanted and 100mL of 1% AS media minus silica with 500 µM $NaNO_3$ was added pre-dawn for 14 days. Predawn samples of 1 mL were collected before and after dilution to measure turbidity, cell count, and NO_x. Cultures were grown on an orbital shaker in 500 mL flasks with 200 mL culture volume under 1000 W metal halide lamps with a photon flux of 200 µmol m^{-2} s^{-1} set to a 14:10 hour L:D cycle, with an ambient temperature of 20 °C. On day 15, triplicate cultures were diluted predawn with 100 mL of 7% AS media to raise the salinity to 4% for the hyper-saline treatment, while the remaining three replicates were diluted with 1% AS media for the isosaline treatment. Salinity was verified using a salinity refractometer. Diurnal samples were collected on day 15 throughout the photoperiod for turbidity, cell count, NO_x, proline content, and BODIPY measurements.

Growth medium. AS medium was prepared according to UTEX culture collection standards with silica omitted [35]. All media were sterilized using a steam autoclave.

Data analysis. Statistically significant subsets for maximum daily specific growth rate and salinity acclimation and shock proline content were determined using an ANOVA with a Tukey's honest standard difference *post hoc* analysis with $\alpha=0.05$.

Assays

Cell counts were performed using a hemocytometer and cell density was calculated using at least 100 counted cells.

Turbidity was measured by aliquoting 300 µL of undiluted culture into 96 well-plates and read at 750 nm with a BioTek H1 Synergy (Winooski, VT) plate reader. Daily specific growth rate (µ) was calculated as follows:

$$\mu = \ln(A_{750} \text{ day n}) - \ln(A_{750} \text{ day n-1})$$

Intracellular proline content - Biomass was pelleted in a centrifuge at 10k relative centrifugal force (RCF) for 10 min, resuspending in 1 mL of 90% ethanol, and stored for at least 24 hrs., but no more than 15 days at -4°C, to extract cellular contents. The ninhydrin reaction [36] and optimized protocol developed by Lee et al. [37] were used to determine proline concentration of the sample, which was converted to attomoles (amol) of proline per cell based on cell density measurements.

NO_x assay - Culture samples were centrifuged at 10k RCF for 10 minutes to pellet cells. Supernatant was removed and analyzed for nitrate plus nitrite content (NO_x) according to Schnetger and Lehners' VCl_3 reduction protocol [38], with standard curves constructed at each salinity to account for the effects of salinity on the reaction.

Flow cytometry and lipid staining - Samples for flow cytometry were pelleted by centrifugation at 10k RCF for 10 minutes. Supernatant was removed and samples were stored at -4° C until analysis, not more than 15 days. Samples were re-suspended in Dulbecco's modified filter-sterilized PBS and flow cytometry measurements were taken using a Becton-Dickinson FACSCalibur on unstained control samples to achieve baseline FL2 readings [39].To stain neutral lipids, samples were incubated in the dark for 10 minutes with 505/515 BODIPY in a DMSO solution to a final concentration of 2 µg BODIPY mL^{-1} sample, and flow cytometry measurements taken again. The difference in median FL2 height between stained and unstained samples was calculated for triplicate cultures to determine neutral lipid content. Flow cytometry data were collected from

10,000 events from each sample and gated for forward scatter and chlorophyll fluorescence for a total of ~7000 events per sample.

CHAPTER III

RESULTS

Growth rate, proline content, and NO_x uptake were assayed in different culture modes and salinity regimes using both *Picochlorum* strains. While the batch culture experiments were designed to investigate the effects of nitrogen on the long term (multiday) salinity shock response, the semicontinuous chemostats studied the short term (single day) salinity shock response in cells previously in steady state.

Batch culture salinity acclimation and shock

To determine the effect of salinity acclimation and salinity shock on growth rate, each strain was grown in iso-, hyper-, and hyposaline batch cultures. Salinity-acclimated *P.* sp. SE3 growth rate decreased with increasing salinity, while *P. oklahomense* grew slightly but not significantly faster when acclimated to 4% than when acclimated to 1% and slowest when acclimated to 7% (Fig 2.). When subjected to hypersaline (1% to 4%) shock, *P.* sp. SE3 grew significantly faster than *P. oklahomense* but there was no difference in growth rate between strains when subjected to hyposaline (7% to 4%) shock. Growth rate did not differ significantly between isosaline cultures of 4% *P.* sp. SE3, 4% *P. oklahomense*, and 1% *P. oklahomense*, as well as hypersaline (1% to 4%)

P. sp. SE3 and both strains under hyposaline (7% to 4%) conditions.

To quantify proline accumulation, samples were taken from batch cultures at 5 and 10 days after inoculation and subjected to the acid-ninhydrin assay as previously described. On day 5 when acclimated to 1% salinity, *P. oklahomense* had around 1 amol proline $cell^{-1}$ and when acclimated to 4% had a mean of around 34 amol proline $cell^{-1}$, but these values did not differ statistically (Fig. 3). Proline content increased significantly when acclimated to 7%, with *P. oklahomense* accumulating over 450 mean amol proline $cell^{-1}$. Compared to *P.* o*klahomense, P.* sp. SE3 accumulated much less proline when acclimated to 7%, but differences between strains were smaller when acclimated to 1% or 4%. In both the hypersaline (1% to 4%) and hyposaline (7% to 4%) treatments, *P.* sp. SE3 accumulated significantly more proline than when it was grown in 4% isosaline media. This differed from *P. oklahomense,* which did not accumulate a significantly different proline content when grown in hyper-, hyper-, and isosaline media. By day 10, proline content for all treatments decreased to near zero, except for *P. oklahomense* acclimated to 7%, which still decreased from day 5 but had a significantly higher proline content than the other treatments on day 10 with a mean of around 96 amol proline $cell^{-1}$ (Fig. 3).

When plotting NO_x assimilated versus cell density, salinity treatments did not differ for *P.* sp. SE3 ($p=0.151$), but *P.* oklahomense did have at least one salinity treatment that had a significantly different relationship between NO_x assimilated and cell density ($p<0.001$) (Supplemental Figure 1).

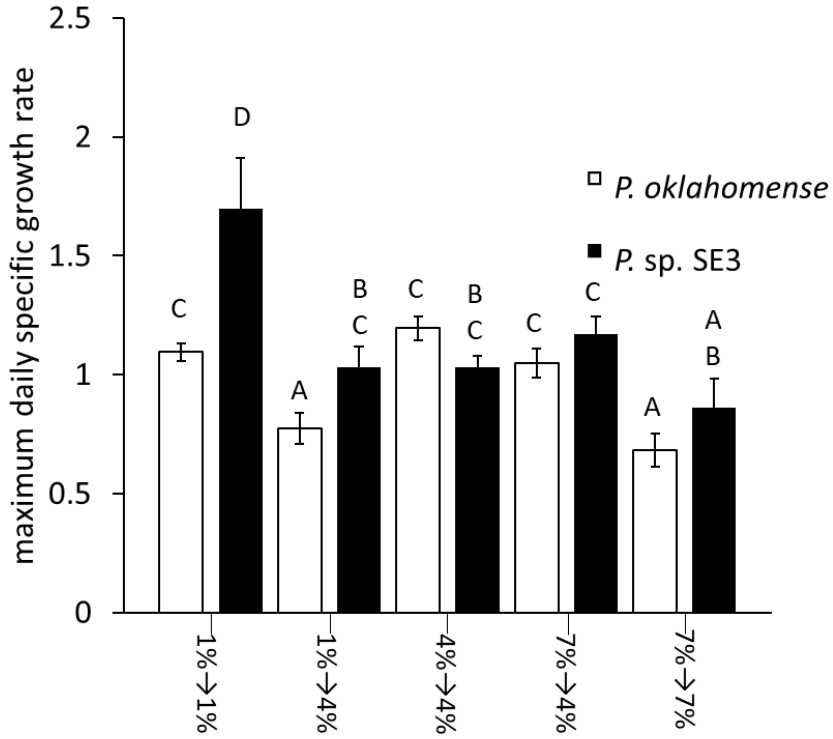

Fig. 2. Batch culture growth rates. Maximum turbidity specific growth rate (μ_{max}) of batch cultures acclimated for >10 generations at 1, 4, and 7% AS inoculated into hypo, iso, and hyper-osmotic media. Letters indicate statistically homogenous subsets (ANOVA and Tukey's honest standard difference test).

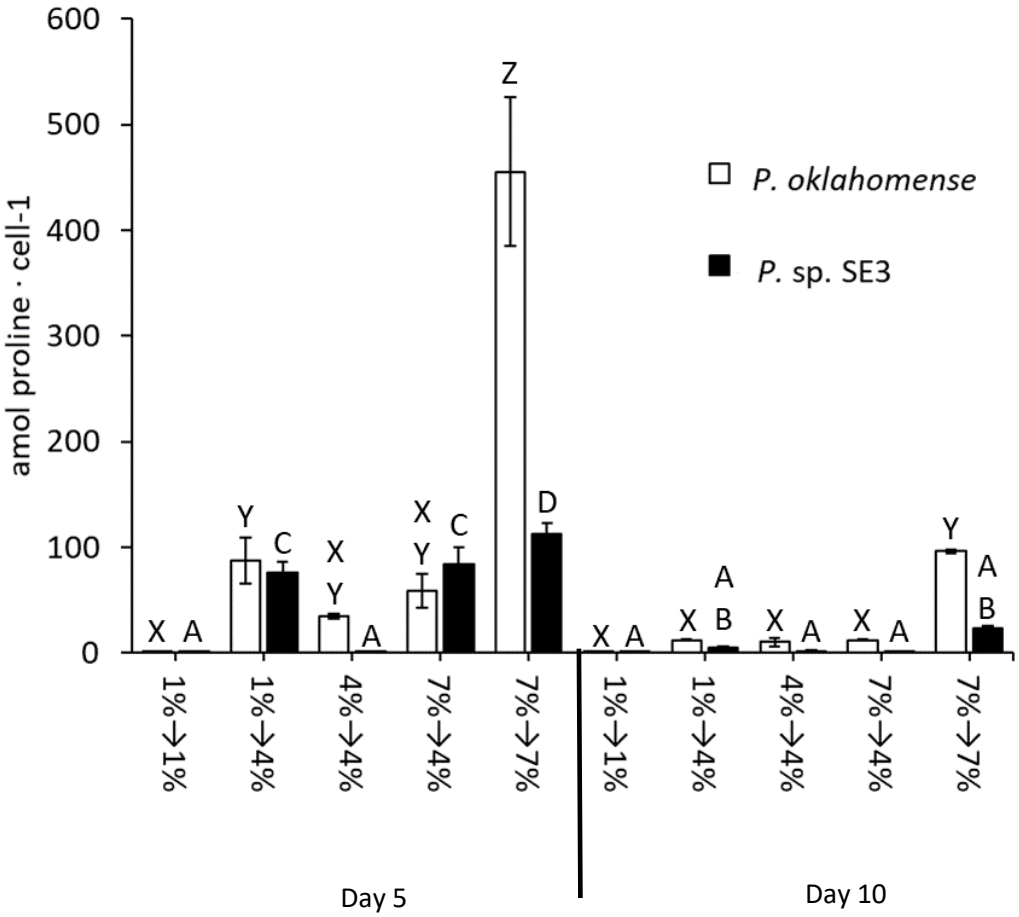

Fig. 3. Batch culture proline content. Salinity acclimated and salinity shock batch culture proline content 5 and 10 days after inoculation. (mean ± SD, n = 3). Letters indicate statistically homogenous subsets within each species (ANOVA and Tukey's honest standard difference test). A-C = *P.* sp. SE3, X-Z = *P. oklahomense*

Semicontinuous culture salinity acclimation and shock

Both strains were grown in semicontinuous chemostat cultures for 14 days at 1% salinity to acclimate to the salinity and culture conditions. On the 15th day, predawn, half of the replicates were transferred into hypersaline media with a salinity of 4% and the other half were transferred into the same isosaline media. Samples were then collected over the course of the photoperiod for iso- and hypersaline treatments in both strains. Turbidity increased as biomass accumulated throughout the photoperiod (Fig. 4A). Strains did not differ when acclimated to 1% AS, with both strains increasing the same amount of turbidity. When shocked with 4% AS, both strains had a smaller increase in turbidity compared with 1% AS, but *P. oklahomense* was more negatively affected than *P.* sp. SE3 and even showed a slight initial decrease in turbidity. Nitrate uptake showed a similar trend, with no difference between strains when acclimated to 1% AS (Fig. 4B). When shocked with 4% AS, nitrate uptake decreased slightly in *P.* sp. SE3 and more sharply in *P. oklahomense*. When acclimated to 1% AS, both strains showed moderate increases in cell density throughout the day, especially late in the photoperiod (Fig. 4C). When shocked with 4% AS, cell densities changed little throughout the photoperiod (Fig. 4C). Neither strain accumulated proline throughout the photoperiod when acclimated to 1% AS (Fig. 4D). Following 4% AS shock, both strains rapidly accumulated proline over the course of the photoperiod, with *P.* sp. SE3 accumulating slightly less proline per cell than *P. oklahomense*.

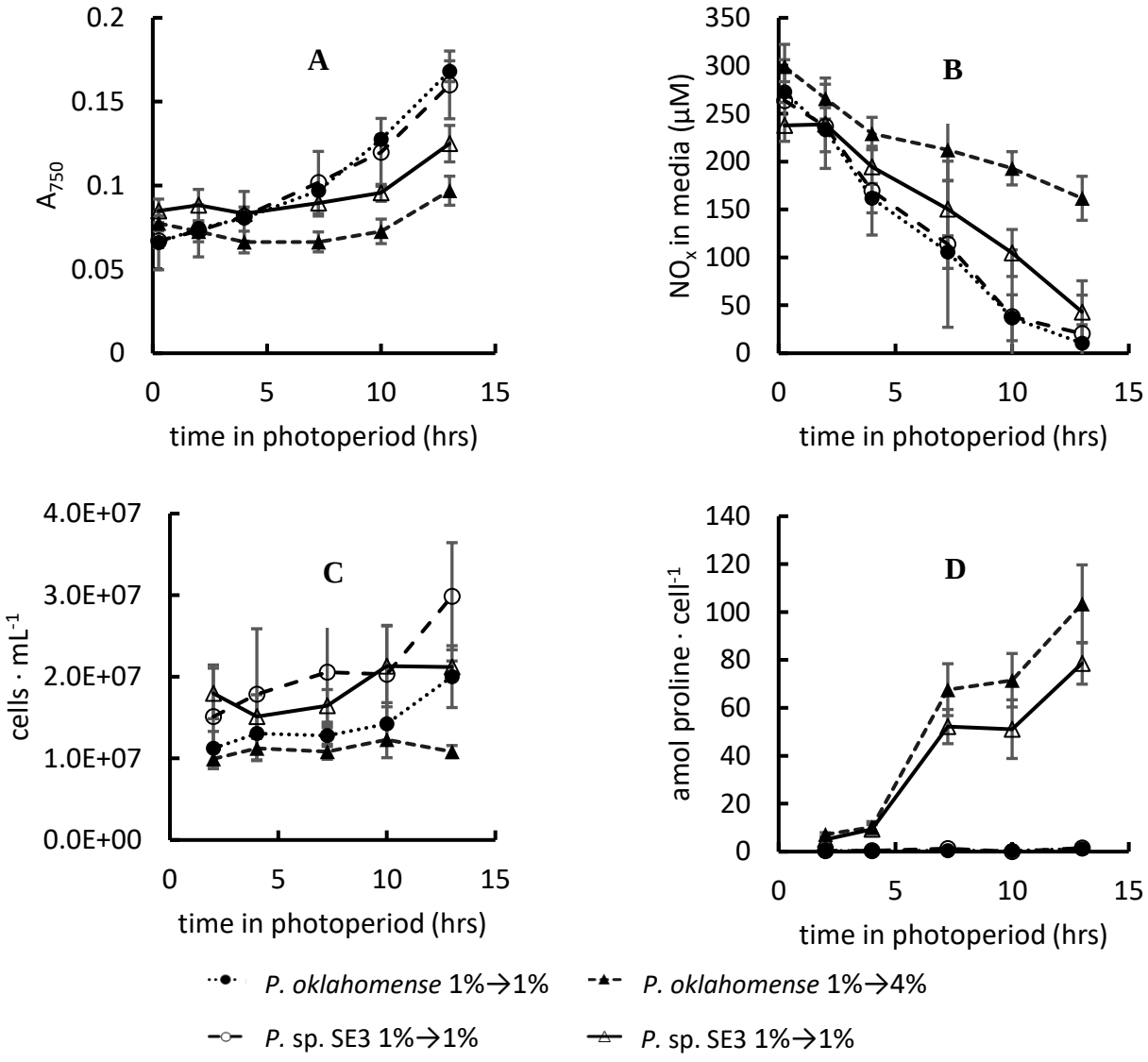

Fig. 4. Semicontinuous culture diurnal data. Day 15 of semicontinuous cultures in salinity acclimation (1%→1 %) and salinity shock (1%→4 %) treatments. (A) Turbidity measured as absorbance at 750 nm. (B) Concentration of cells in culture. (C) Concentration of NO_x in culture. D shows proline content per cell. Error bars represent standard deviation of triplicate cultures.

Neutral lipid content of the *P. oklahomense* semicontinuous 1% isosaline control culture decreased early in the photoperiod and increased late in the day, while the hypersaline treatment showed less of a decrease over the photoperiod and a smaller increase late in the day (Fig. 5). The *P.* sp. SE3 1% control cultures showed a steady lipid content over the photoperiod, while the hypersaline (1% to 4%) shocked cultures showed a similar steady trend throughout the photoperiod but with a slight increase late in the day. *P. oklahomense* hyper saline shocked cells had a greater lipid content than all other treatments at most time points. Figure 6 shows the detailed flow cytometry data from 10 hours post-dawn, when the *P. oklahomense* hypersaline treatment showed a higher lipid content. Lipid content is determined by the difference between the stained and unstained samples, with a larger difference indicating a higher lipid content. Each panel in figure 6 shows the stained and unstained triplicate samples merged onto the same plot to illustrate the difference between the unstained, lower fluorescing cloud and the stained, higher fluorescing cloud.

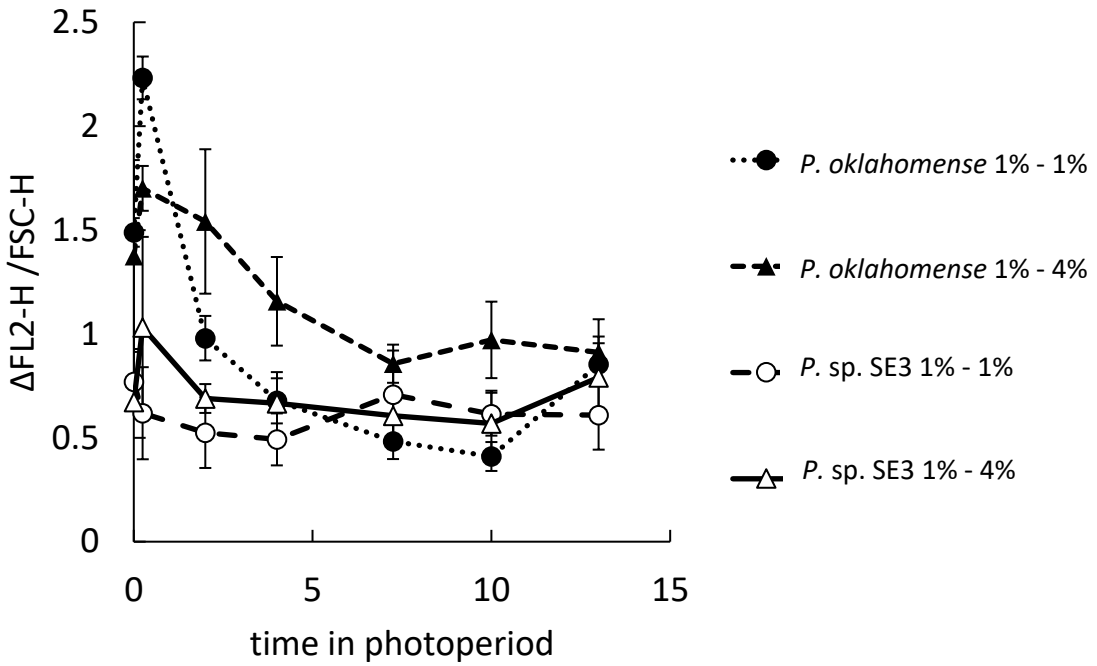

Fig. 5. Semicontinuous culture lipid content. Cell size-normalized BODIPY fluorescence (ΔFL2/FSC) over the course of the photoperiod on day 15 of triplicate semicontinuous chemostat cultures (mean ± SD).

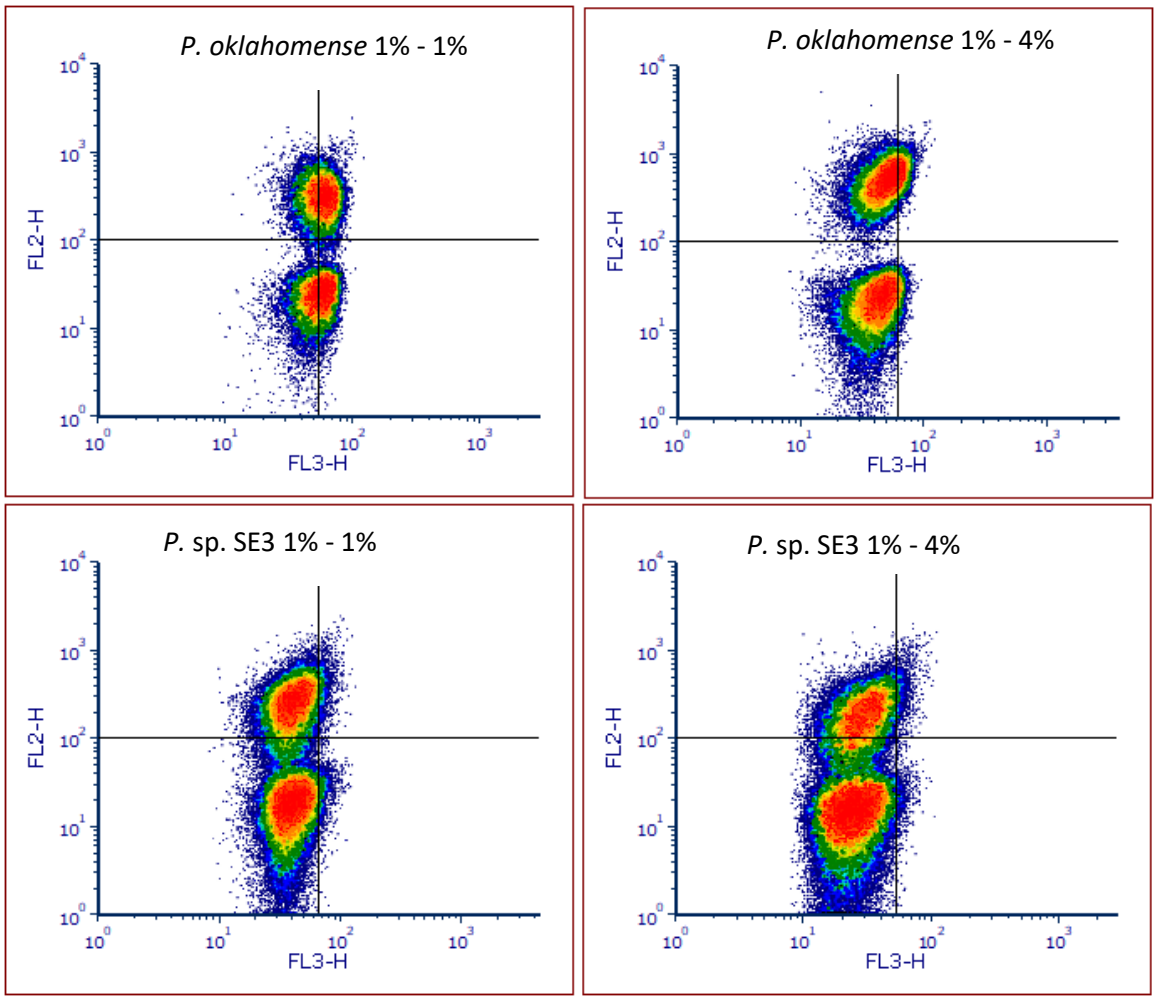

Fig. 6. Flow cytometry visualization. Results from day 15 of semi-continuous culture, 10 hours into the photoperiod. Each panel represents merged samples of triplicate cultures before and after staining with BODIPY, with the difference in FL2-H indicating fluorescence from lipid-BODIPY complexes. Larger differences between stained and unstained samples indicate greater lipid content (note $\log_{10}$ scale). FL3-H represents chlorophyll autofluorescence and was used to exclude non-algal contaminants from the analysis.

CHAPTER IV

DISCUSSION

These experiments show the difference in growth between salinity-acclimated and salinity-shocked cultures in two closely related strains. We studied this under constantly changing physiological state in batch cultures, and initially steady state physiology in semicontinuous cultures. The two strains were isolated from distinct poikilohaline habitats, but their history of residence in and adaptation to those habitats is unknown. Nevertheless, they possess distinct differences in genome organization despite nearly identical 18s rRNA sequences, and thus may be expected to exhibit physiological differences [31, 32].

Based on the batch culture salinity shock experiment, *P.* sp. SE3 responds better to rapid changes in salinity. It grows faster after both hypo- and hypersaline shock compared to *P. oklahomense*. This is perhaps made possible by the organization of the *P.* sp. SE3 genome into functional gene clusters, with genes encoding enzymes involved in a shared metabolic pathway being co-located in the genome, allowing faster or more efficient activation of stress response mechanisms [31]. Utilization of proline under different steady state salinities differs between strains, consistent with transcriptomic

evidence showing that proline synthesis is upregulated under increasing salinity [19]. It is apparent that *P. oklahomense* relies heavily on proline accumulation to acclimate to high salinity, while *P.* sp. SE3 accumulates significantly less proline at high salinity and does not accumulate proline at all at moderate (4% or approximately marine) salinity. This is interesting because it suggests that the genomic differences (horizontal gene transfer events, genome organization) present in *P.* sp. SE3 confer additional salinity tolerance mechanisms that *P. oklahomense* lacks, therefore necessitating the increased reliance on proline in *P. oklahomense* when acclimated to high salinity. However, both strains accumulate similar levels of proline when subjected to both hypo- and hypersaline shock. This supports past research that shows that proline is used for more than just balancing the osmotic pressure between the intracellular and extracellular environment, and may be involved in stabilizing proteins, scavenging reactive oxygen species and stabilizing free radicals produced during hypo- and hypersaline shock similarly in both strains, but is used differently for salinity acclimated growth [21]. The decrease in proline content from day 5 to day 10 is also interesting because it suggests that as the cultures deplete nitrogen in the media, nitrogen in the cells may be recovered from free proline. Future transcriptomic or metabolomic analyses and nutrient limitation studies investigating a macronutrient other than nitrogen would help to show whether proline degradation is dependent on nitrogen availability or growth stage.

Results of the semi-continuous experiment can be used to understand the short-term responses to perturbation with cells initially in steady state physiology and growth rate. In semi-continuous cultures, cells grow at a constant exponential rate, assimilating nutrients in stable proportion to photosynthesis and cell division. Operated as a

chemostat, the same level of nutrients is supplied each morning, allowing the cultures to achieve a steady biomass density from day to day. As in batch growth, both strains grew slower following hypersaline shock, but more so in *P. oklahomense* than in *P.* sp. SE3. The slower increase in turbidity mirrors the slower assimilation of nitrogen seen after hypersaline shock, with *P.* sp. SE3 having a faster N uptake rate than *P. oklahomense*. Both strains accumulate proline at a similar rate, but when proline accumulation is plotted against nitrogen uptake, *P. oklahomense* accumulates significantly more proline despite significantly lower nitrogen uptake rate compared to *P.* sp. SE3 (Suppl. Fig. 2).

Neutral lipid accumulation differs more between isosaline and hypersaline shock treatments in *P. oklahomense* than in P. sp. SE3. This suggests that *P. oklahomense* is more stressed following hypersaline shock, while *P.* sp. SE3 is better adapted to salinity shock and does not accumulate lipids in response to short-term hypersaline shock. This illustrates a tradeoff that plagues the algae biofuel production strategy: higher levels of physiological stress may raise intracellular lipid content, but comes at a cost of decreased overall productivity in both the short- and long-term.

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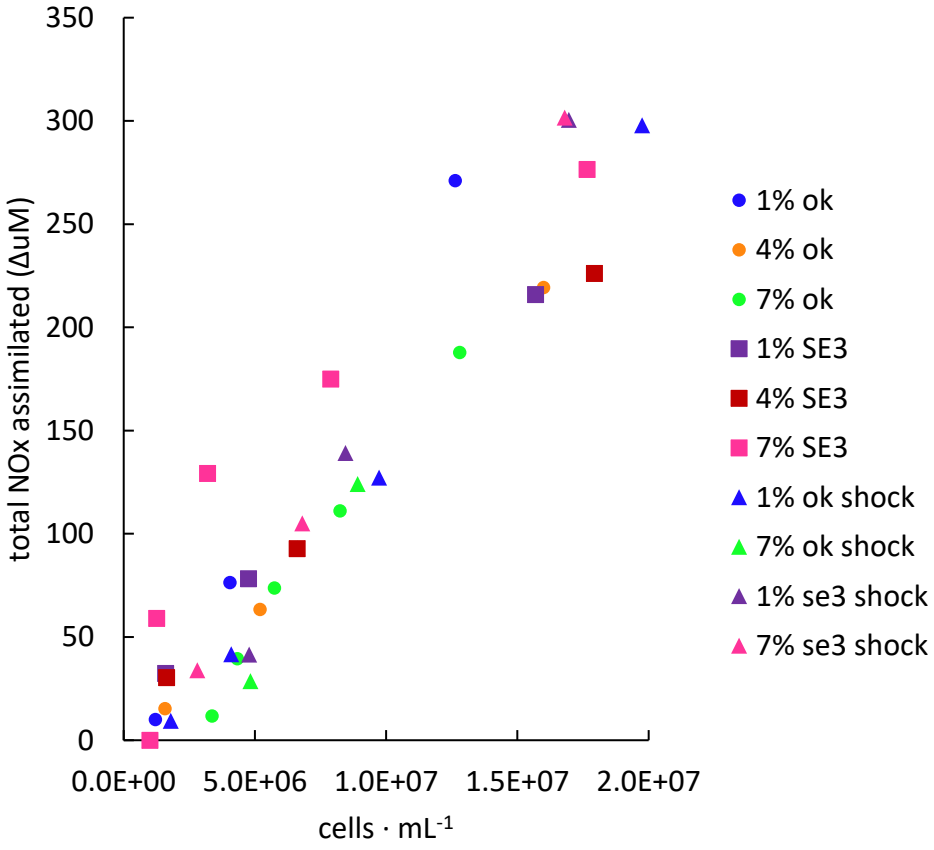
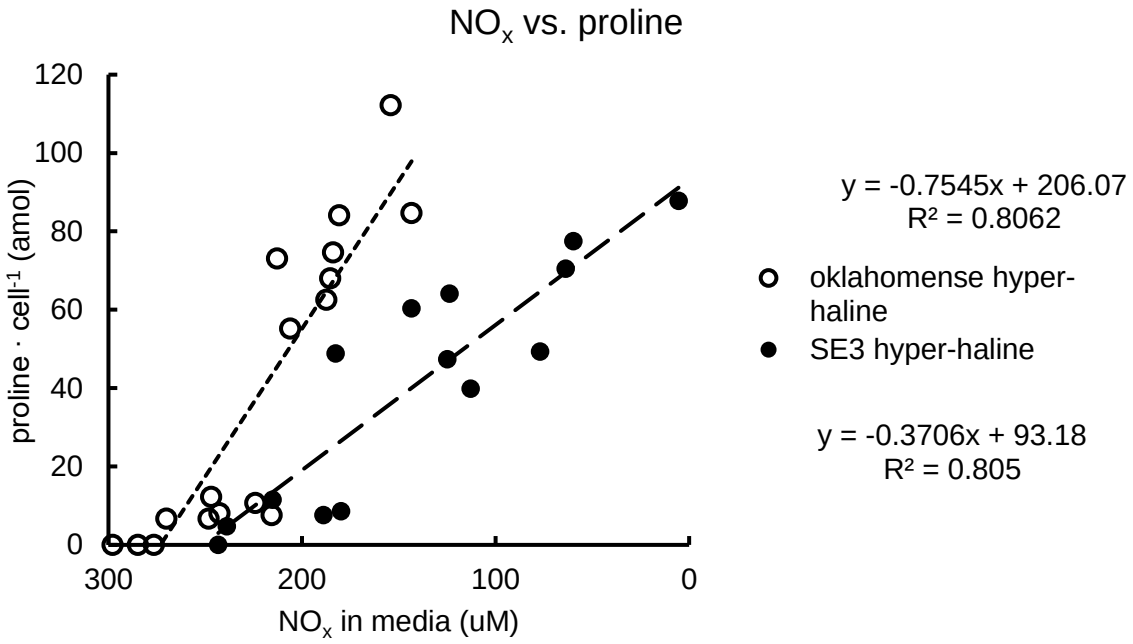
APPENDICES

Supplemental figures



Supplemental Figure 1. NO_x uptake vs cell density from batch culture iso-, hypo-, and hypersaline treatments. Analysis of covariance (ANCOVA) found no significant difference between treatments of *P.* sp. SE3 ($p=0.151$), but *P.* oklahomense had at least one significantly different treatment ($p < 0.001$).



Supplemental Figure 2. NO_x in media versus proline content in semi-continuous chemostat cultures.

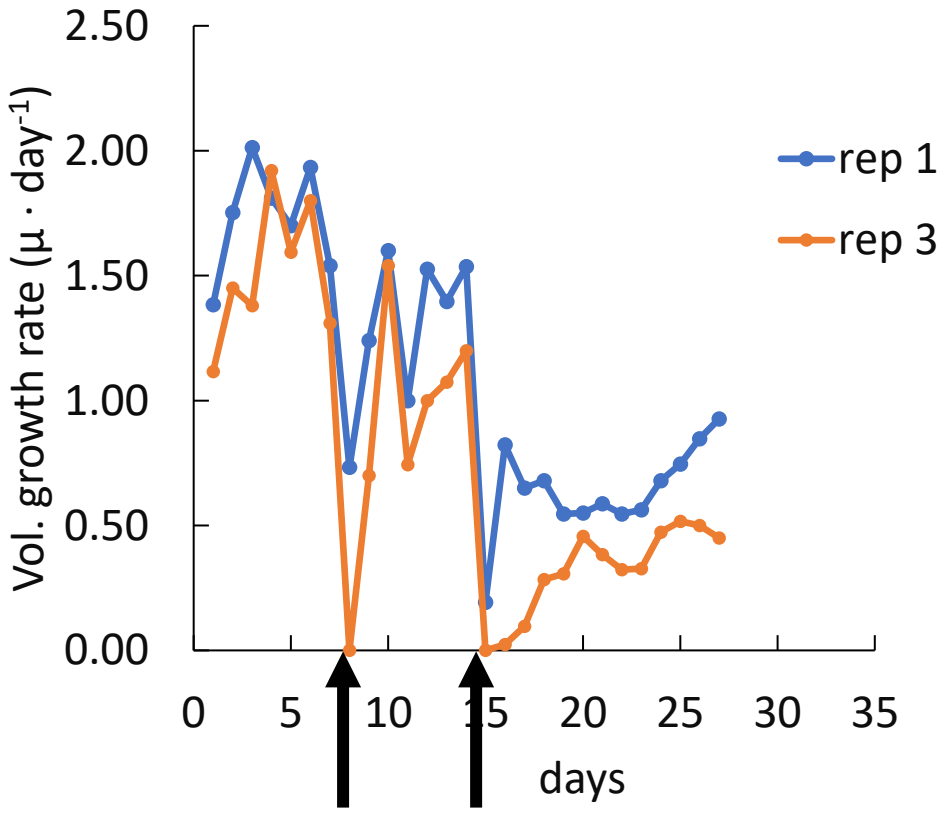
Turbidostat experiment

While batch cultures provide a single initial supply of N that is used up over time and semicontinuous chemostats supply N once per day, turbidostats provide N in much smaller increments spaced across the photoperiod. This method allows for less variability in the growth rate of unperturbed cultures because as turbidity increases, N is continually added, preventing the cells from ever experiencing N limitation and maintaining a constant biomass density and growth throughout the photoperiod and from day to day, thus eliminating the confounding effect of nitrogen depletion on growth rate seen in batch and semicontinuous cultures. This turbidostat experiment examines multi-day, nutrient replete responses to hypersaline shock.

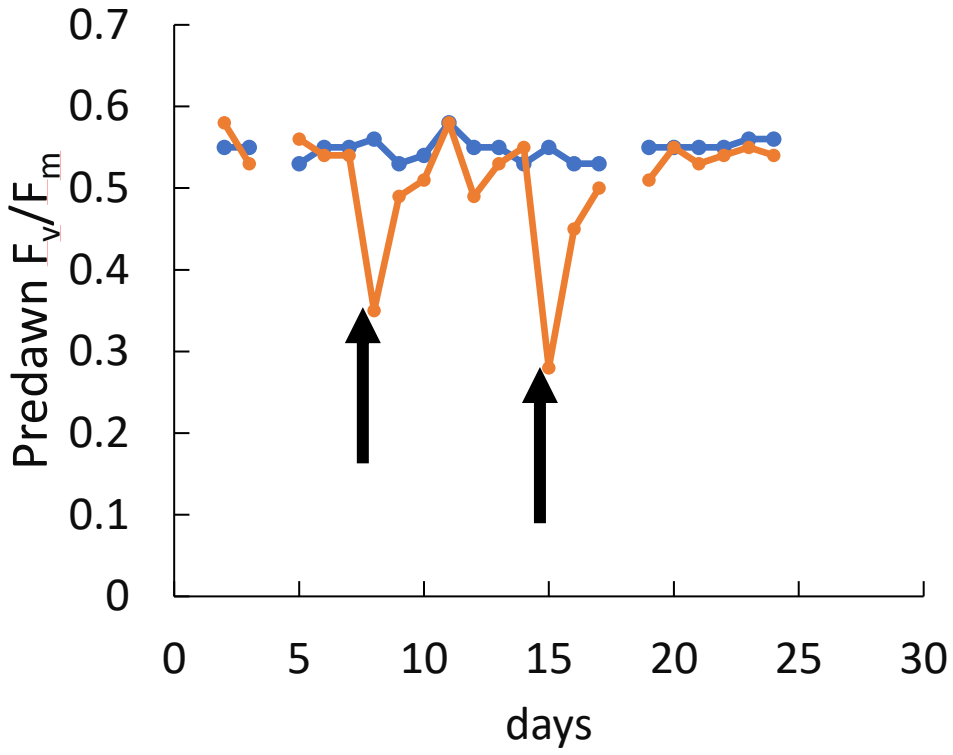
P. oklahomense was grown in the cycloturbidostat system previously described in [13, 40] with a 14-hour photoperiod under red/white/blue LEDs with a photon flux of 600 µmol m^{-2} s^{-1}. Two replicates were supplied with AS media containing 600 µM $NaNO_3$ at 1% salinity and grown in the turbidostat for 13 days. On day 14, 50mL of culture was removed and replaced with 50mL of filter sterilized 19% salinity AS to bring salinity of the cultures to 4%, and influent media was replaced with 4% AS containing 600 µM $NaNO_3$. Cultures were grown in turbidostat mode for 6 days and on day 21, 50 ml of each culture was removed and replaced with 50 mL of filter sterilized 22% AS to bring salinity of the cultures to 7%, and influent media was replaced with 7% AS containing 600 µM $NaNO_3$. Cultures were then grown in turbidostat mode for 10 days. Daily, predawn 8-mL samples were collected and used for turbidity and rapid light curve measurements. Samples were collected for proline content assays at multiple timepoints (Supp. Fig. 6).

There was considerable variation between replicates, this may be due to slight differences in initial cell density that manifested in large observable differences in most variables. Both replicates exhibited an obvious decrease in growth rate following hypersaline shock, with recovery to below pre-shocked levels in 2-3 days (Supp. Fig. 3). Predawn F_v/F_m decreased for one replicate following hypersaline shock but recovered rapidly within 1 day to pre-shocked levels (Supp. Fig. 4). The other replicate may have been in a better physiological condition and able to recover in less than one day, before the next predawn measurement was taken, preventing a transient decrease from being observed. The maximum relative electron transport rate ($rETR_{max}$) reacted similarly to F_v/F_m, but both replicates showed a decrease in $rETR_{max}$ following hypersaline shock with a recovery to pre-shocked levels within 1-2 days (Supp. Fig. 5). These rapid light curve parameters indicate that hypersaline shock stresses the photosystem, but after acclimation normal processes resume. The cultures rapidly accumulated proline following 4% hypersaline shock and again after shock to 7%, and maintained high proline content for the duration of the experiment (Supp. Fig. 6). This contrasts with the

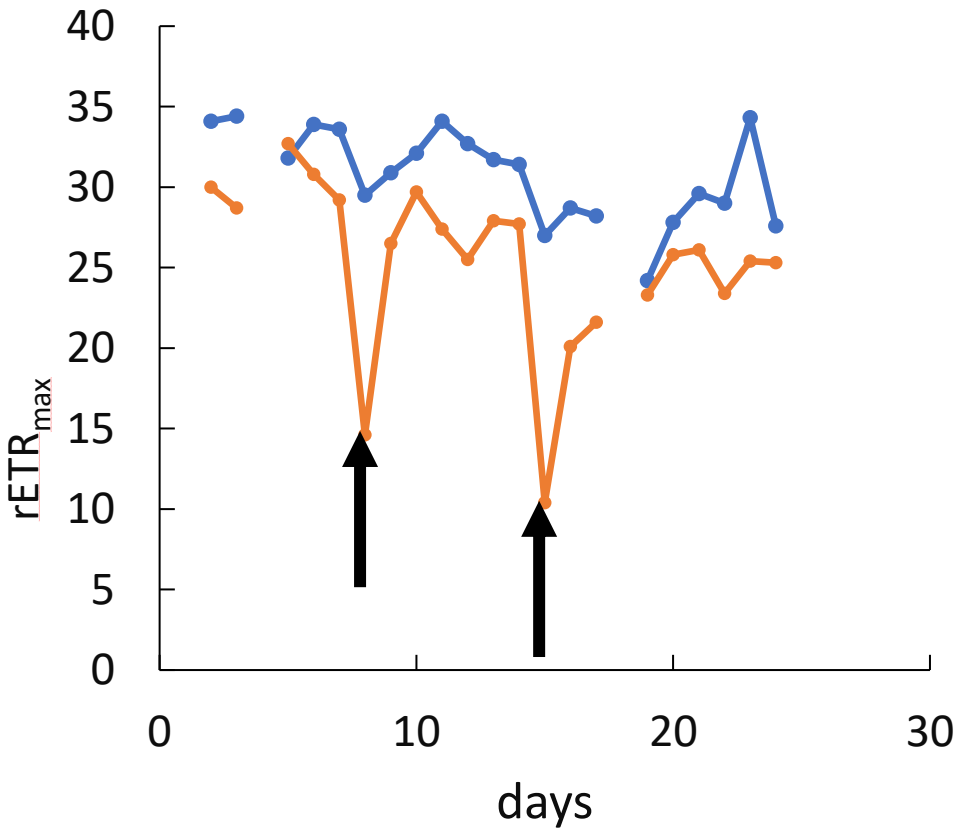
batch culture experiment where proline decreased from day 5 to day 10, and shows that proline degradation does not occur in fast growing, nitrogen-replete cultures.



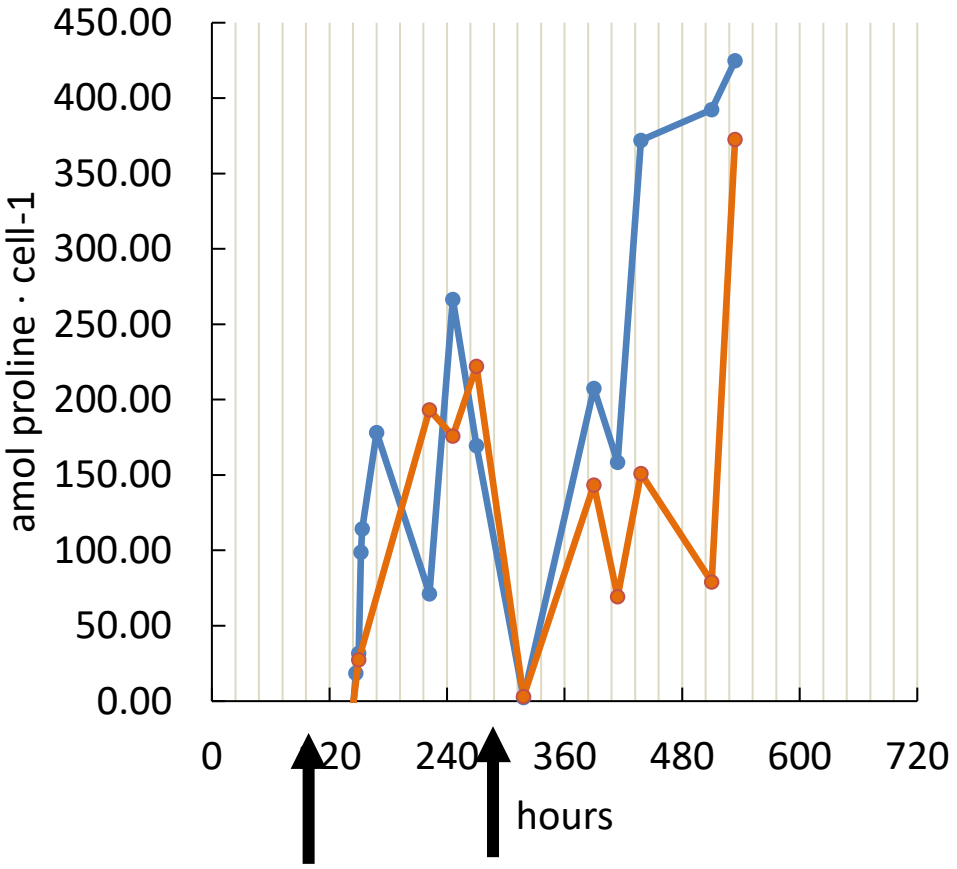
Supplemental Figure 3. Volumetric growth rate of replicates 1 and 3 of *P. oklahomense* grown in cycloturbidostat culture mode. Arrows indicate when hyper saline shock was applied pre-dawn, first arrow is 1% to 4% AS, second arrow is 4% to 7% AS.



Supplemental Figure 4. Pre-dawn maximum potential quantum efficiency of PSII (F_v/F_m) of replicates 1 and 3 of *P. oklahomense* grown in cycloturbidostat culture mode. Arrows indicate when hyper saline shock was applied pre-dawn, first arrow is 1% to 4% AS, second arrow is 4% to 7% AS.



Supplemental Figure 5. Pre-dawn maximum relative electrom transport rate ($rETR_{max}$) of replicates 1 and 3 of *P. oklahomense* grown in cycloturbidostat culture mode. Arrows indicate when hyper saline shock was applied pre-dawn, first arrow is 1% to 4% AS, second arrow is 4% to 7% AS.



Supplemental Figure 6. Free proline content of replicates 1 and 3 of *P. oklahomense* grown in cycloturbidostat culture mode. Arrows indicate when hyper saline shock was applied pre-dawn, first arrow is 1% to 4% AS, second arrow is 4% to 7% AS. Vertical lines indicate days.

VITA

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Master of Science

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Completed the requirements for the Associate of Science in Biology at Southwestern Illinois College, Belleville, Illinois in 2011.

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Assayed algal physiology using flow cytometry, chlorophyll fluorescence, light microscopy, and elemental analysis

Professional Memberships:

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Algae Biomass Organization