**The unexpected extremophile: tolerance to fluctuating salinity in the green
alga *Picochlorum***

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Abstract

The broadly halotolerant green alga, *Picochlorum* strain SENEW3, has a highly reduced nuclear genome of 13.5 Mbp that encodes only 7,367 genes. It was originally isolated from a shallow, mesophilic brackish-water lagoon that experiences extreme changes in temperature, light, and in particular, salinity (freshwater to 3-fold seawater). We challenged *Picochlorum* cells with high or low salinity shock and used transcriptomic and chlorophyll fluorescence analyses to elucidate tolerance to salinity fluctuation. The transcriptome analysis showed that one-half of the coding regions are differentially expressed in response to salinity changes. In addition, a significant number of co-expressed genes (usually from different metabolic pathways) are co-localized in the genome, forming 2-10 gene clusters. Whereas the overall salt stress response in *Picochlorum* SENEW3 is similar to that in other salt-tolerant algae, the “operon-like” structure in this species likely contributes to rapid recovery during salinity fluctuation. In summary, our work elucidates how evolutionary forces play out in a streamlined genome. *Picochlorum* SENEW3 relies on a broad array of adaptations from the reliance on horizontally transferred adaptive genes to the co-localization of stress response genes and a robust photosystem II to deal with a fluctuating environment. These attributes make *Picochlorum* SENEW3 of great biotechnological interest.

Keywords: *Picochlorum*; *Chlorophyta*; halotolerance; salt stress; co-localization; RNA-seq

Abbreviations: HGT, horizontal gene transfer; DE, differentially expressed; ASW, artificial seawater; FRRF, fast repetition rate fluorometer; STF, single turnover flash; PSII, photosystem II; WOC, water-oxidizing complex; QF, quality factor; FDR, false discovery rate; L₂fc, log₂ fold change; TCA, tricarboxylic acid cycle; THF, tetrahydrofolate; LIN, lincomycin.

1. Introduction

Most well known eukaryotic extremophiles are better described as polyextremophiles because they survive multiple types of environmental stress. The sea ice diatom, *Melosira arctica*, faces not only cold temperatures but also high pH, irradiance stress, and fluctuating salinity due to the brine habitat in the ice (Boetius *et al.*, 2013). On the other end of the temperature scale, *Galdieria sulphuraria* and *Cyanidioschyzon merolae*, red algal thermoacidophiles native to hot spring environments, tolerate high levels of salinity and toxic metal concentrations as well as low pH (Barbier *et al.*, 2005). What is shared by these species are highly reduced genomes and gene inventories (5-10 thousand genes) and functional specialization (Wolf & Koonin, 2013). In the case of *G. sulphuraria*, horizontal gene transfer (HGT) and expansion of gene families with adaptive functions also have played key roles in their evolutionary history (Qiu *et al.*, 2013; Schönknecht *et al.*, 2013). But are there environments that are less exotic (i.e., mesophilic) that may pose just as much environmental risk? These habitats could include the intertidal zone and shallow water lagoons, both of which endure high ultraviolet and visible light levels, desiccation, and fluctuating salinity. To address this issue, we studied *Picochlorum* sp. strain SENEW3 (SE3) (hereafter, *Picochlorum* SE3), a “polyextremotolerant” green alga (Chlorophyta, Trebouxiophyceae) that was isolated from a shallow water estuary in San Diego County, California. This alga has one of the smallest genomes known (13.5 Mbp) for a free-living eukaryote and encodes only 7,367 genes (Foflonker *et al.*, 2015). Nonetheless, *Picochlorum* SE3 is remarkably robust in the face of environmental perturbations, thriving in freshwater as well as in 3-fold the salinity of seawater, light intensities between 80–2000 $\mu\text{E}/\text{m}^2/\text{s}$, and temperatures that range from at least 16–33°C (Wang *et al.*, 2014). *Picochlorum* SE3 shows similarities in halotolerance range, environment, and other

abiotic stress tolerances to the closely related species *Picochlorum oklahomensis* isolated from the Great Salt Plains of Oklahoma (Henley *et al.*, 2007).

Several other *Picochlorum* species including *P. oklahomensis* and *P. atomus*, have been investigated and are considered suitable for biofuel, nutritional, nutraceutical, and waste water remediation applications (De la Vega *et al.*, 2011; Chen *et al.*, 2012; Zhu, 2012; El-Kassas, 2013; Pereira *et al.*, 2013; von Alvensleben *et al.*, 2013; Zhu & Dunford, 2013; 2014; Dahmen *et al.*, 2014). This is due to the robustness, ability to harvest through common flocculation methods (Zhu, 2012), high biomass production (1.8-2.1 g/L maximum biomass) (De la Vega *et al.*, 2011; Zhu & Dunford, 2013), high protein content (350-550 g/kg) (von Alvensleben *et al.*, 2013; Zhu & Dunford, 2013; Dahmen *et al.*, 2014), high carotenoid content (De la Vega *et al.*, 2011), and lipid accumulation (total lipid content reported to be 16-25% dry weight, unstressed cells) (De la Vega *et al.*, 2011; Pereira *et al.*, 2013; Zhu & Dunford, 2013; Dahmen *et al.*, 2014) exhibited by members of this genus. The NAABB consortium reported a *Picochlorum* strain with the ability to rapidly accumulate lipids under nitrogen depletion, accompanied with an increase in starch accumulation, and demonstrated effective genetic manipulation in increasing lipid accumulation by as much as 38% (Department of Energy, 2014). Salinity has also been reported as an effective method of crop protection in reducing freshwater cyanobacterial contaminants in *P. atomus* cultures (von Alvensleben *et al.*, 2013). Therefore, *Picochlorum* SE3 may be of great biotechnological interest due to its ability to withstand a hypervariable environment. This allows the use of salinity as a crop protection mechanism and makes this alga potentially suitable for large-scale open-pond cultivation.

2. Materials and Methods

2.1. Salinity shock experimental conditions

Picochlorum sp. strain SE3 was cultivated in artificial seawater (Goldman & McCarthy, 1978) based Guillard's f/2 medium (Guillard & Ryther, 1962) containing 1M NaCl, without silica (f/2 ASW -Si). Cultures were grown at 25° C under continuous light (100 $\mu\text{E m}^{-2} \text{s}^{-1}$) on a rotary shaker at 100 rpm (Innova 43, New Brunswick Eppendorf). To determine growth rate, cells adapted to 1M NaCl f/2 media were inoculated in f/2 media containing various salt concentrations and cell counts were performed using a hemacytometer (Neubauer improved, Hausser Scientific), image capture (Infinity 2 camera, Lumenera corporation), and ImageJ counting software. Growth rates were determined based on cell counts during exponential phase using triplicate cultures (Equation 1).

$$\text{growth rate} = \frac{2.303(\log(\text{count}_2) - \log(\text{count}_1))}{(\text{time}_2 - \text{time}_1)} \quad (1)$$

For the transcriptome experiment, cells adapted to 1M NaCl were pelleted, washed, and inoculated in fresh f/2 media containing 1.5M NaCl (high salt stress) and 10mM NaCl (low salt stress). NaCl was the only component of the f/2 ASW -Si media modified for all experiments. Approximately 100 mg of cells were harvested and flash frozen after 1h and 5h of incubation with salt treatment under 100 $\mu\text{E m}^{-2} \text{s}^{-1}$ light. Cells harvested prior to treatment were used as the control. This experiment was performed in triplicate; i.e., three separate cultures were used for each salinity condition and each of these was sampled at the 1h and at 5h time points.

2.2. Transcriptome sequencing

Frozen algal cell samples were homogenized using the TissueLyser II (Qiagen) and total RNA was extracted according to the RNeasy Plant Mini Kit (Qiagen) protocol. The cDNA libraries were constructed using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA) following the manufacturer's protocols. RNA concentrations were determined using a NanoDrop 2000c Spectrophotometer (Thermoscientific) and Qubit 2.0 Fluorometer (Life Technologies). Libraries were sequenced using the MiSeq Personal Genome Sequencer (Illumina) with 2x80bp (paired end) and 1x160bp read lengths. See Table S1 for details. These Illumina transcriptome data can be retrieved via NCBI BioProject RJNA245752 and are also available at <http://cyanophora.rutgers.edu/picochlorum/>.

2.3. Transcriptome analysis

RNA-seq reads were trimmed and aligned to the *Picochlorum* SE3 genome using CLC Genomics Workbench. Reads < 50bp in length were discarded during trimming and the RNA-seq analysis in CLC was performed using default parameters. Differential expression was determined using DESeq (R/bioconductor package) (Love *et al.*, 2014) and read counts determined by the CLC RNA-seq analysis as the input. A *P*-value of 1% and a Log₂ fold change of 1 were set as the cutoffs for all differentially expressed (DE) genes discussed in this paper. DE genes were used as input for the KEGG metabolic pathway mapping tool (Kanehisa *et al.*, 2011) to identify metabolic impacts of salinity stress. Gene ontology (GO) term enrichment analysis was performed using the Fisher's exact test as part of the blast2go software (Conesa *et al.*, 2005). Differential gene expression overlap between treatments was determined with Venny 2.0 (Oliveros, 2007). The inference of protein targeting was done using TargetP (Emanuelsson *et al.*, 2000).

2.4. Co-localization analysis

A co-regulated gene cluster was defined as two or more genes with shared properties (e.g., up-regulation in response to salt treatment) that were physically linked. One or two intervening genes (without the shared properties) between adjacent genes were allowed. Gene clusters were merged when separated by two genes or less. To test if the observed clustering of genes up-regulated by salt treatment was significantly more than expected by chance alone, we removed genes derived from contigs encoding less than three genes. Clusters of up-regulated genes were then identified as described above. We randomly sampled the same number of genes as those actually up-regulated from the total gene population, and identified gene clusters using this randomly sampled gene set. This random sampling-based analysis was repeated 1,000 times and the information regarding resulting gene clusters was recorded. The gene cluster information (the number of clusters and number of genes in clusters) derived from actual data and randomly generated data were plotted as shown in supplementary information (SI) Fig. S4. The p -value of actual gene number in a cluster (or cluster number) was defined as the number of random samples generating a clustered gene number equal to or greater than the actual number divided by the total random sample size (i.e., 1000).

2.5. Photosynthetic measurements

Chlorophyll fluorescence was measured in Fig. 1 using a fast repetition rate fluorometer (FRRF) with saturated light pulses produced by a laser diode at an intensity of $32,000 \mu\text{E m}^{-2} \text{s}^{-1}$ with a $50\mu\text{s}$ flash duration (defined as single turnover flash, STF), after 2 minutes of dark incubation (Ananyev & Dismukes, 2005). Mid-exponential phase cells adapted to 1M NaCl were

161 pelleted then shocked in media with various salinities. Aliquots and measurements were taken
162 hourly. The relative quantum efficiency of PSII charge separation (QY) was approximated by the
163 yield of variable chlorophyll fluorescence intensity (F_v/F_m) (Kolber *et al.*, 1998). Signal
164 averaging of four trains of 50 flashes was performed with 2 min dark pre-incubation between
165 each train. The steady-state value of F_v/F_m was obtained by averaging the first 50 individual
166 STFs, denoted F_v/F_m , and is reported herein for three biological replicates. This average
167 eliminates the transient damping of period-four oscillations.

168 The relative fraction of photosystem II water-oxidizing complex (PSII-WOC) centers that
169 achieve productive water oxidation following primary charge separation was determined from
170 fits of the amplitude of the period-four oscillations of F_v/F_m from individual STFs. The
171 oscillations from 50 STFs were fitted to a modified Kok model (VZAD model) to obtain the
172 inefficiency parameters describing the rate of damping and the S state populations in the dark
173 prior to flashing (Vinyard *et al.*, 2013). By analogy with resonance circuit theory, the Quality
174 Factor (QF) is used to quantify the efficiency of oscillations, defined as the inverse of the Kok
175 damping terms, $QF = (\alpha + \beta)^{-1}$. Here, α represents misses (STF not resulting in
176 advancement of oxidation state of PSII-WOC) and β represents double hits (STF resulting in
177 advancement by two oxidation states). Kok parameters were determined as an average of four
178 measurements. The QF is reported as an average of three biological replicates.

179 Photoinhibition was determined by tracking F_v/F_m during exposure to $1500 \mu E m^{-2} s^{-1}$
180 high light conditions in the presence and absence of chloroplast protein synthesis inhibitor
181 lincomycin (LIN). Exponential phase cells adapted to 1M NaCl were pelleted and then shocked
182 with various salinities with the addition of 10 mM bicarbonate and 1 mM LIN. F_v/F_m was
183 measured using a PAM (pulse amplitude modulated) fluorometer (Photon Systems Instruments,

Brno, Czech Republic) after 2-minute dark adaptation. Samples were exposed to high light for a total of 70 minutes with measurements taken every ten minutes (7 min light exposure, 2 min dark adaptation, 1 minute measurement time). Experiments were performed with three biological triplicates. The PSII inactivation rate was calculated by fitting F_v/F_m over time to a biexponential decay function using the OriginPro 2014 software (OriginLab). The lifetimes of the biexponential curves were calculated using the fitting parameters (Fig. 4) and equation 2:

$$lifetime = (A1 \times t1) + (A2 + t2) \quad (2)$$

3. Results and Discussion

3.1. High and low salinity stress elicits separate metabolic responses

We determined the appropriate conditions to study short-term response to salt stress in *Picochlorum* SE3. The growth rate and average PSII quantum efficiency (proportional to F_v/F_m) were used to assess the effects of various salt concentrations on 1M NaCl pre-adapted cultures of the alga (Fig. 1). The time dependence of F_v/F_m over 24 h shows that the PSII quantum yield, which is a measure of the light energy conversion to heat + photochemical water oxidation, is diminished at both lower and higher salt concentrations. The salt dependence of F_v/F_m after 24 h follows a similar sequence as the growth rate, indicating that energy resources that normally go into growth are diverted to the maintenance of osmotic balance, with both hypo- and hyper-osmotic balance diverting energy resources. Higher growth rates at lower salinities (0.1 and 0.01M) than the pre-adapted condition (1M) may simply indicate that lower salinity results in optimum growth over the period of several days. However, Fig. 1 indicates that lower salinities have similar effects as high salinities in the short-term. When examined in more detail it is apparent that the kinetic response of F_v/F_m differs for hypo- vs. hyper-osmotic changes. The

kinetics of recovery are faster when the change in salt concentration is smallest for both hypo- and hyper-osmotic changes. Additionally, all of the hyper-salinity samples (1M, 1.5M, and 1.6 M NaCl) attain a steady-state between 7h and 24 h, whereas the hypo-salinity samples (0.1M, 0.01M, and 0 M NaCl) are still recovering at 24 h, indicated by the positive slopes. This difference may be simply because the changes in concentration are so much larger (10X and 100X), thereby slowing recovery. No large-scale cell rupture due to osmotic shock was observed microscopically, but smaller-scale rupture could have gone unnoticed due to the small cell size (2-3 μm) of this coccoid alga.

For the transcriptome analysis we chose 1.5M and 10mM NaCl concentrations to represent hyper- and hypo-salinity stresses because both elicited similar photosynthetic efficiency responses in the short-term and exhibited some recovery of growth rate under long-term acclimation. The 1M to 10mM stress represents what *Picochlorum* cells might experience during a rain event in its natural environment. Transcriptomic analysis reveals that salt stress induces significant gene expression changes in *Picochlorum* SE3 with a total of 3,681 genes (50% of the nuclear gene inventory) being differentially expressed (DE; $>1 \log_2$ fold change [hereafter, $L_2\text{fc}$]), $p < 0.01$, adjusted for the false discovery rate, FDR, of 5%) by salt stress over the 5-hour time course, relative to the pre-stress control. This includes 12 of 24 genes that originated in *Picochlorum* SE3 via HGT from bacterial sources (SI Table S2) (Foflonker *et al.*, 2015), suggesting functional relevance of these foreign genes in salt tolerance, analogous to what was found in *G. sulphuraria* (Qiu *et al.*, 2013; Schönknecht *et al.*, 2013). More genes showed differential expression at 1h (3,256) than at 5h (1,629) and around one-half of the DE genes at 1h returned to below threshold levels (<1 and $<-1 L_2\text{fc}$) by the 5h time point (77% high, 68% low salinity) (SI Fig. S1, S2). In addition, each salinity treatment resulted in different DE gene sets,

with some statistically significant overlap of genes involved in multiple treatments (SI Fig. S1, Table S3; the full list of DE genes is shown SI Table S4). For example, the 1h up-regulated gene set shared 173 genes, with 742 and 722 genes belonging uniquely to the high and low salinity responses, respectively. In terms of biochemical pathways, there is little overlap in the initial high and low salinity responses, indicating two very different types of stress on the cell (Fig. 3, SI Fig. S3). We posit that high and low salinity shock, and salt acclimation all represent different challenges to *Picochlorum* SE3, which deals with them using specialized gene sets.

3.2. Co-localization of co-expressed genes in response to salt shock

For each culture treatment, we searched for clusters of co-localized DE genes with a shared expression pattern (i.e., co-expressed), allowing for a maximum of two intervening genes not sharing that expression pattern (Fig. 2a). Randomization analysis was performed to determine the statistical significance of these putative clusters (SI Fig. S4). These results indicate that the number of genes in clusters is statistically significant in most data sets, meaning that larger clusters of co-localized genes are found within co-expressed data sets than could be attributed to chance alone. Under some conditions, the number of clusters formed is also significant. These results hold true for the majority of the conditions, even when more stringent gene expression cutoffs are used (1.5 vs. 1.0 L₂fc) (SI Table S5, Fig. 2a). Allowing for three intervening genes resulted in loss of statistical significance in clustering. The underlying transcription control mechanisms of these clusters are, however, unknown.

The co-localized genes comprise 2-10 gene clusters and constitute 42-72% of the total number of genes in individual co-expressed data sets (1.0 L₂fc cutoff). The results shown in Fig. 2 demonstrate that more co-expressed genes, both up- and down-regulated and at both salinities,

are co-localized at 1h when compared to the 5h time point. For example, 61.6% (546/886) of genes form clusters under high salinity at 1h compared to 42.4% (225/531) at the 5h time point (1.0 L₂fc cutoff). This suggests that genes are organized in close proximity in the genome for more efficient regulation of gene expression during the initial phase of salinity shock. Although these clusters of co-localized and co-expressed genes are functionally related in that they all take part in the salt stress response, the vast majority do not appear to be members of the same biochemical pathway. Exceptions include genes involved in urea degradation, nitrate assimilation, acetate assimilation, pyruvate to acetoin conversion, some light regulated genes, and photorespiration (SI Table S6). Interestingly, many of the co-localized genes in *Picochlorum* SE3 are not physically linked in the genomes of other green algae such as *Chlorella vulgaris* and *Coccomyxa subellipsoidea* (SI Table S6; full output presented in SI Table S7). Some clusters, including genes in the nitrate assimilation pathway, however, are partially conserved in other green algae including, *C. vulgaris*, *C. subellipsoidea*, and *C. reinhardtii* (Quesada *et al.*, 1998). The cluster of photorespiration related genes appear to be conserved in *C. subellipsoidea* as well. These results suggest that stress-related genes in streamlined eukaryotic genomes may be organized in operon-like structures similar to bacteria. This presumably allows rapid activation under stressful conditions. However, no evidence of polycistronic transcription was found in *Picochlorum* SE3.

3.3. High photorespiration influences carbon and nitrogen flux at high salinity

The most striking change in response to high salinity shock at 1h is the up-regulation of genes involved in photorespiration (Fig. 3; SI Table S8), which may function in stress protection (Wingler *et al.*, 2000). Photorespiration in green algae such as *C. reinhardtii* differs from that in

plants, in that glycolate is converted to glyoxylate *via* glycolate dehydrogenase in the mitochondrion rather than *via* glycolate oxidase in the peroxisome (Stabenau & Winkler, 2005). In traditional photorespiration, H₂O₂ is a byproduct of the glycolate oxidase reaction, resulting in an increased need for catalase activity. Up-regulation of glycolate dehydrogenase and down-regulation of glycolate oxidase and catalase suggests that the mechanism of photorespiration in *Picochlorum* SE3 is similar to that of other green algae. Glycolate dehydrogenase is also located in a cluster with two other genes in the photorespiration pathway (SI Table S6).

Reduced CO₂ concentrations at high salinities can inhibit the preferred photosynthetic carbon fixation pathway of Rubisco, stimulating the competing pathway of photorespiration. Conditions of CO₂ limitation are also correlated with increased carbon concentrating activities *via* up-regulation of carbonic anhydrase in *Dunaliella salina*; i.e., to increase photosynthetic carbon fixation through the Calvin-Benson-Bassham cycle (Booth & Beardall, 1991; Fisher *et al.*, 1996). *Picochlorum* SE3 also displays this response with strong up-regulation of carbonic anhydrase under high salinity stress. Carbon flux through the TCA cycle is down-regulated at 1h, potentially due to the inhibition of reductant production *via* the TCA cycle due to excessive NADH produced during the glycine to serine conversion in photorespiration (Bykova *et al.*, 2005) (Fig. 3, SI Fig. S5). One-carbon metabolism including tetrahydrofolate (THF) synthesis from glycine and the interconversion between its derivatives is also highly up-regulated under high salinity. In *Arabidopsis*, THF metabolism is essential to photorespiration, thereby maintaining carbon fixation under CO₂-limited conditions (Collakova *et al.*, 2008).

High photorespiration rates in plants result in the generation of ammonia as a byproduct of the conversion of glycine to serine, which is subsequently converted to glutamate (Rachmilevitch *et al.*, 2004). Accordingly, one copy each of glutamine synthetase and

ferrodoxin-dependent glutamate synthase, the major pathway for ammonia fixation, is up-regulated in *Picochlorum* SE3 under high salinity stress. Nitrate and urea assimilation are also significantly up-regulated, potentially serving as nitrogen sources for the increased protein synthesis demands under salinity stress. The opposite is true for nitrogen assimilation at low salinity stress, however, translation and ribosome biogenesis are up-regulated. These results again indicate that *Picochlorum* SE3 cells are less stressed at low (versus high) salinity.

Starch and osmolytes

Proline is the major osmolyte in *Picochlorum oklahomensis*; glycerol and glucosylglycerol were detected to a lesser extent (Henley *et al.*, 2004). One gene involved in proline synthesis was up-regulated in *Picochlorum* SE3 under high salinity, and an increased expression of pathways leading towards glutamate synthesis, the precursor of proline, was observed. In *Dunaliella*, starch formation decreases and starch degradation increases as salinity increases in favor of glycerol synthesis (Goyal, 2007; Xia *et al.*, 2014). In contrast, starch synthesis is up-regulated and starch degradation is down-regulated, whereas no evidence of increased glycerol synthesis was observed in *Picochlorum* SE3 under the high salt conditions used in this experiment. Synthesis of trehalose, another osmolyte, is up-regulated at 1h and sorbitol is up-regulated at 5h. As expected, in order to maintain osmotic equilibrium at low salinity, osmolyte synthesis is down-regulated, and accordingly starch synthesis is up-regulated. The up-regulation of starch synthesis under both high and low salinity stress conditions may indicate that proline rather than glycerol is the major osmolyte in *Picochlorum* SE3. Other aspects of the stress response in *Picochlorum* SE3 (e.g., cell wall and membrane remodeling) are discussed in the supplementary information Notes S1.

3.4. Response of the photosynthetic machinery

Photoinhibition involves light-induced oxidative damage to the PSII protein D1 and inactivation of the WOC (Murata *et al.*, 2007; Kolling *et al.*, 2009). PSII repair entails D1 protein digestion by internal proteases and replacement of the damaged protein via *de novo* synthesis (Dasgupta *et al.*, 2008). Photoinhibition, when accelerated by environmental stresses, including salinity, reduces photosynthetic carbon fixation, which accelerates light-driven H₂O₂ generation, which in turn accelerates D1 protein damage (Neale & Melis, 1989; Allakhverdiev & Murata, 2004; Takahashi & Murata, 2008; Kolling *et al.*, 2009). We measured the rate of photoinhibition under high light, 1500 $\mu\text{E m}^{-2} \text{s}^{-1}$ (i.e., different from the transcriptome experimental conditions), at various salinities in the presence and absence of the chloroplast protein synthesis inhibitor lincomycin (LIN). These photoinhibition curves (Fig. 4) are the result of several interacting phenomena: photoprotection, D1 biosynthesis *de novo*, and osmoregulation after salinity shock. They may be fitted via biexponential decrease of F_v/F_m , with the two phases distinguished by an initial period of osmoregulation and a later phase dominated by D1 biosynthesis. Both high and low salinity shock conditions exhibit a similar pattern of reduced decline of D1 protein synthesis compared to the control (1M NaCl); 10 mM NaCl shows the shortest difference in lifetimes between samples with and without LIN (1.6 min) (Fig. 4a) compared to the control (16.9 min), 1.5M NaCl (6.1 min), and 0.4M NaCl (6.8 min) conditions. We postulate that the additional stress of salinity shock diverts resources from D1 protein repair to the energy intensive process of maintaining cell homeostasis during salt shock. These results partially support the hypothesis that high salinity may provide protection against other abiotic stresses such as irradiance and temperature. This phenomenon has been reported for other

halotolerant green algae, such as *Nannochloris* sp. and *Dunaliella parva* (Henley *et al.*, 2002). However, because our experiments examined salinity shock, the observed lessened stress effects under 0.4M and 1M NaCl and high light may be better explained by acclimation.

SI Fig. S7 highlights the faster recovery of the WOC cycling efficiency, indicated by the quality factor (QF) at low salinity shock versus high salinity shock (halftime = 0.5 h at 10mM NaCl and 4.5 h at 1.5 M NaCl). Recovery of WOC cycling efficiency appears to correlate with recovery of F_v/F_m or overall photosynthetic efficiency at high salinity, but recovers faster than F_v/F_m at low salinity. Whereas, D1 repair is equally inhibited by the low and high salinity conditions, WOC efficiency may provide a greater contribution to photodamage at high salinities.

4. Conclusions

Our genome-wide and PSII analyses demonstrate that despite living in an apparently mesophilic lagoon environment, environmental fluctuations have left significant “footprints” on the *Picochlorum* SE3 genome. Diametrically opposed salinity conditions impose distinct challenges that this alga responds to with specialized gene sets. Shock and acclimated responses also differ, highlighting the challenges posed by a rapid versus gradual environmental change with respect to salinity. Overall, *Picochlorum* SE3 responds to high/low salinity stress in a similar fashion as other algae and plants; i.e., photoprotective mechanisms, oxidative stress response, cell wall and membrane rearrangement, nitrogen assimilation, and diverting resources from growth and PSII repair in favor of maintaining homeostasis. Despite these shared responses, the key to the ability of *Picochlorum* SE3 to withstand massive environmental fluctuations is likely explained by genome organization. Co-localization of genes within

specialized gene sets appears to function similarly to bacterial operons in enhancing a rapid response to shock. In summary, our study highlights a compact genome that has evolved a broad array of adaptations from HGT to co-localization of stress response genes to a robust PSII to deal with a challenging environment. Although our results suggest that energetic resources are diverted from growth and productivity during periods of salinity shock in favor of maintaining cell homeostasis, *Picochlorum* SE3 is highly adapted to rapid acclimation (beginning within 5 hours of salinity shock) to salinity shock and maintains growth rates over a broad range of salinities. Therefore, *Picochlorum* SE3 may be suitable for open-pond cultivation under conditions of high irradiance and high salinity, which would not be tolerable to competing freshwater and many marine microalgal species. Its broad range of salinity and shock tolerance makes *Picochlorum* SE3 particularly suited to cultivation in seawater or saline groundwater because it can tolerate evaporative loss in an open-pond environment.

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Figure Legends

Fig. 1. (a) Average chlorophyll variable fluorescence yield (F_v/F_m) and (b) Growth rate of the algal cultures acclimated to 1M NaCl media, following transfer to media containing various salt concentrations under $100 \mu\text{E m}^{-2} \text{s}^{-1}$ light. F_v/F_m is the average of 4 trains of 50 STFs and three biological replicates. Error bars represent standard deviation.

Fig. 2. (a) Examples of co-expressed and co-localized gene clusters. Gene clusters, as defined in the text, are denoted with boxes. Blue represents up-regulated genes; red, down-regulated; gray, not DE. The numbers above the boxes show relative gene locations within contigs; gene numbers

are labeled above the boxes. (b) Number of genes co-localized versus total genes in gene set (percentages labeled) at the 1.0 and 1.5 L₂fc cutoffs. Only statistically significant results are shown (see SI Table S5 for details).

Fig. 3. Summary of the salt shock response in *Picochlorum* SE3 at 1h under (a) high salinity and (b) low salinity conditions. Genes/pathways in blue are up-regulated (>1.0 L₂fc); red, down-regulated (< -1.0 L₂fc); gray, not DE (<1.0 L₂fc and > -1.0 L₂fc). Solid colored arrows indicate that at least one copy of the gene is DE (i.e. other copies may not be DE), whereas arrows with a blue/red gradient have gene copies that are both up- and down-regulated. The numbers correspond to enzymes shown in Table S8. G3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; DHA, dihydroxyacetone; THF, tetrahydrofolate; SQDG, sulfoquinovosyl diacylglycerol; UDP-GlcNAc, uridine diphosphate N-acetylglucosamine; geranylgeranyl-PP, geranylgeranyl pyrophosphate.

Fig. 4. Photoinhibition under 1500 $\mu\text{E m}^{-2} \text{s}^{-1}$ high light conditions in the presence and absence of chloroplast protein synthesis inhibitor lincomycin (LIN). Cells adapted to 1M NaCl media were incubated in media at various salinities. The data are fit to a second order exponential decay curve. The relative amplitudes (A1, A2) and lifetimes in minutes (t1, t2) values are shown. Error bars represent standard deviation.

The following **Supporting Information** is available for this article:

Notes S1. Salinity stress response in *Picochlorum* SE3

548 **Fig. S1.** Venn diagram comparing differentially expressed genes at both salinities and time
549 points

550 **Fig. S2.** Gene expression patterns at high and low salinity over the time course

551 **Fig. S3.** KEGG metabolic map overview comparing the low and high salinity stress response

552 **Fig. S4.** Example of a randomization analysis that simulates gene clustering.

553 **Fig. S5.** KEGG pathway analysis of genes involved in the TCA cycle comparing high and low
554 salinity shock

555 **Fig. S6.** KEGG pathway analysis of genes involved in protein processing in the endoplasmic
556 reticulum comparing high and low salinity shock

557 **Fig. S7.** Effect of salinity on quality factor (QF)

558 **Table S1.** Number of RNA-seq reads sequenced.

559 **Table S2.** Differentially expressed genes of bacterial origin.

560 **Table S3.** Excel file of shared differentially expressed genes between the four conditions tested.

561 **Table S4.** Excel file of all DE genes. Highlighted cells (orange) denote HGT.

562 **Table S5.** Summary of number of co-expressed co-localized genes forming clusters.

563 **Table S6.** Examples of co-expressed co-localized gene clusters

564 **Table S7.** Excel file of all co-expressed co-localized genes.

565 **Table S8.** Accompanying table for Fig. 3 with gene expression values of differentially expressed
566 genes.

567

Figure 1

Figure 2

Figure 3

(a)

(b)

Figure 4