TRANSCRIPTOMIC RESPONSE OF *Karenia brevis* TO ENVIRONMENTAL STRESS

A Thesis

by

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ABSTRACT

Karenia brevis is a toxic marine phytoplankton that forms harmful algal blooms in the Gulf of Mexico. This toxicity is due to the production of ladder-frame polyketides known as brevetoxins, for which the production pathway and function in the cell are not known. Osmotic stress has been shown to increase the production of brevetoxin in *K. brevis*, and recent studies implicate the toxin as linked to the function of light harvesting complex II in the thylakoid membrane. Understanding whether brevetoxin production and photosynthetic stress are linked in an osmotic shock response will shed further light on its function in the cell. Using *K. brevis* clones with high, average, low, and non-detectable levels of brevetoxin, we explored the response to osmotic stress using analysis of differential gene expression, measurements of PAM fluorometry, and production of the known osmolyte glycerol. Brevetoxin-producing clones of *K. brevis* showed lower expression in Photosystem II genes, and correspondingly lower photosynthetic efficiencies and intracellular glycerol concentrations in response to stress. In contrast, clones that produce non-detectable and low toxin levels showed no significant stress response, and also showed a deficiency in Non-Photochemical Quenching (NPQ) compared to toxic cultures. This observation supports the potential link between reactive oxygen species and NPQ pathways that is related to the presence of brevetoxin in *K. brevis*. Further, brevetoxin-producing *K. brevis* clones respond to osmotic stress by utilizing photosynthesis for osmoacclimation and through small molecule degradation, in particular intracellular photosynthetically derived glycerol, whereas the low and non-detectable clones do not.

DEDICATION

This thesis is dedicated to every person who helped make it possible. Thank you.

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1. INTRODUCTION

K. brevis is an autotrophic marine dinoflagellate that produces neurotoxic ladder frame polyketides called brevetoxins (Baden, 1989). The ecological and molecular function of brevetoxin in the *K. brevis* cell is not known. Recently, brevetoxin production has been shown to increase in response to hypo-osmotic stress in *K. brevis* clones that ordinarily produce normal to high amounts of brevetoxin (8–18 $pg \cdot cell^{-1}$). In contrast, a low-toxin producing clones (2–4 $pg \cdot cell^{-1}$) did not show this response, which suggested a link between environmental conditions and brevetoxin production in the cell (Errera & Campbell, 2012). Moreover, another recent study demonstrated that brevetoxin is localized in the thylakoid membrane of chloroplasts, and associated with the light harvesting complex II of photosynthesis (LHCII) (Cassell *et al.*, 2015). Cassell and co-workers found that the non-photochemical quenching (NPQ) capabilities and the amounts of reactive oxygen species (ROS) were significantly different among the *K. brevis* Wilson clones with normal *vs.* low levels of brevetoxin production. Comparisons with *K. brevis* clones of varying brevetoxin production due to stress suggest that the role of the toxin is tied to the cell's response to osmotic stress.

Mechanisms underlying the physiological adaptations algae use to acclimate to osmotic stress are poorly understood (Dittami *et al.*, 2017). It appears that photosynthetic processes and osmotic shock responses are closely linked to each other, as osmoacclimation in algae can involve the use of NPQ to regulate photosynthesis (Nitschke *et al.*, 2010). NPQ is the process of high light energy dissipation as heat,

where photosynthetic processes are reduced in response to this quenching from a conformational change in PSII (Horton & Ruban, 2005). NPQ can be induced by the presence of reactive oxygen species, where the more stressed cells have larger amounts of ROS by accumulation of these oxygen species in Photosystems I and II (Yamori *et al.*, 2016). This ROS signal to induce NPQ can be due to many environmental factors such as high light conditions, osmotic stress, heat stress, and ionic stress (Song *et al.*, 2013, Silva *et al.*, 2011). As ROS increase in response to stress, NPQ is employed by the cell to dissipate excess energy and decrease the accumulation of ROS from photosynthetic processes (Cassell *et al.*, 2015). ROS accumulation in response to stress, inducement of NPQ in response to this accumulation, and finally a corresponding decrease in photosynthetic capacities have been observed in *Arabidopsis thaliana* under heavy metal stress, *Arabidopsis* under excess light and hydrogen peroxide stress, in sunflowers under drought stress, and in light-stressed *Chlamydomonas* (Maksymiec *et al.*, 2007, Karpinski *et al.*, 1999, Maury *et al.*, 1996, Peers *et al.*, 2009).

Regulating photosynthesis has two advantages for osmoacclimation, it can limit the accumulation of ROS by Photosystems I and II and reduce the production of the small molecules from the Calvin Cycle that are osmotically active (Kirst, 1990). Studies on the haptophyte *Prymnesium parvum* have shown changes in the genes galactolipid galactosyltransferase (upregulated) and monogalactosyldiacylglycerol (MGDG-downregulated) that correspond to photosynthetic pathways being transcriptionally regulated in a direct osmotic response (Talarski *et al.*, 2016). The brown alga *Ectocarpus* also showed a transcriptional response to osmotic stress with photosynthetic pathway

transcriptional activations, including a response in photosynthetic antenna genes under low salinity stress (Dittami *et al.*, 2009).

Glycerol is a common small molecule degraded by algae under hypo-osmotic stress; specifically photosynthetic glycerol is degraded intracellularly and exuded under hypo-osmotic stress in dinoflagellates (Suescún-Bolívar & Thomé, 2015, Suescún-Bolívar *et al.*, 2016). Glycerol is degraded in a photosynthetic response to stressors by glycerol dehydrogenase and dihydroxyacetone kinase into dihydroxyacetone phosphate (DHAP), and during osmoacclimation, chloroplast-specific isoforms of the enzymes are used for the conversions (Borowitzka & Brown, 1974, Goyal, 2007a). In response to osmotic dilution stress, *Dunaliella* degraded photosynthetic glycerol into DHAP to osmoacclimate, and during salt stress *Dunaliella* used photosynthetic carbon and the breakdown of starch molecules to synthesize glycerol (Goyal, 2007b). The observed changes in glycerol concentration from osmotic stress is well supported as a major osmoregulatory response pathway, in species such as the green alga *Chlamydomonas* that showed an increase in production of intracellular glycerol in a response that linearly correlated with increasing salinity (Ahmad & Hellebust, 1986).

K. brevis osmoacclimation related to photosynthesis could be influenced by the presence or production of brevetoxin in the cell. Cassell *et al.* (2015) measured an NPQ deficiency in a low brevetoxin-producing clone of *K. brevis* compared to a normal toxin-producing clone, suggesting that brevetoxins could play a role in NPQ and further in a photosynthetic stress response. Because brevetoxin is localized to the thylakoid

membrane where it interacts with LHCII, it has been hypothesized that the presence of brevetoxin is a catalyst for the conformational change in Photosystem II during NPQ in *K. brevis*. An exact mechanism for this catalyst function was not known or proposed, but based upon the NPQ results Cassell *et al.* (2015) proposed that levels of brevetoxin and the conformational change during NPQ were directly related. Cassell and coworkers proposed two alternative scenarios as well; brevetoxin activates an ion channel in the thylakoid membrane, and brevetoxin is used to assemble and form ion channels that promote cation transport in the thylakoid membrane. Specifically, Cassell *et al.* (2015) found that a low toxin-producing *K. brevis* clone had a high amount of ROS compared to its Wilson counterpart that produced normal levels of brevetoxin. This higher ROS content and NPQ-deficiency measured in this particular *K. brevis* clone suggests the low toxin-producing Wilson clone may be defective in photosynthetic capacities due to a lack of brevetoxin.

Variation in the amounts and profiles of brevetoxin produced by *K. brevis* has been shown in laboratory cultures, with no observed correlation between cell growth rate and amount of brevetoxin produced (Errera *et al.*, 2010). The toxic clone Wilson (hereafter, called TW) produces normal amounts of brevetoxin, the TXB4 clone produces relatively high amounts of brevetoxin, and the SP1 clone produces low, but detectable amounts of brevetoxin. Errera *et al.* (2012) specifically found an increase in brevetoxin in *K. brevis* clones TW and TXB4 under osmotic stress. Our laboratory recently observed a new Wilson variant that produces no detectable brevetoxin (hereafter, called NT). This loss of brevetoxin production presented an opportunity to find differential responses to

environmental stress connected with brevetoxin presence and abundance in the cell. Following the recent results (Errera & Campbell, 2012, Cassell *et al.*, 2015), we hypothesized that 1: NT is defective in response to osmotic shock compared to TW; and 2: Less than normal brevetoxin production prevents a response to hypo-osmotic stress in *K. brevis* clones.

Differential gene expression can provide insights into how algal cells respond at the molecular level to acclimate to stress. Here we report measurements of the *K. brevis* transcriptomic response to hypo-osmotic stress. Our objectives were to identify important genes in the hypo-osmotic stress response of *K. brevis* and to corroborate these gene expression trends with physiological assays. Time course experiments were designed to take simultaneous mRNA and toxin samples after osmotic stress to identify a stress response and the recovery response until the point of acclimation. Physiological assays looking at photosynthetic capacities and glycerol concentrations following the same time points were performed to support identified trends in transcriptomics.

2. METHODS

To examine the response of the dinoflagellate *K. brevis* to salinity stress, two strains of brevetoxin producing *K. brevis*, TW (CCFWC268) and TXB4 (CCFWC267), and two low-toxin producing strains, NT Wilson and SP1, were grown in L1-medium made from seawater collected offshore in the Gulf of Mexico adjusted to salinity of 35. Seawater was filtered and sterilized before enrichment via filter-sterilized nutrient addition. Cultures were maintained at irradiance levels of 70 μmol photons $m^{-2} \cdot s^{-1}$ on a 12:12 hour light:dark cycle with cool white bulbs inside a 20˚C Precision Scientific Dual Program Illuminated 818 Incubator. Growth rates were determined from daily cell counts using a Sedgwick-rafter counting chamber and an Olympus BX60 microscope (100×).

K. brevis control and stress cultures were grown in triplicate in 500mL of culture volume and harvested at late logarithmic growth phase (monitored by microscopic cell counts) as per Errera & Campbell 2012. Hypo-osmotic stress was induced by diluting cultures to a salinity of 27 to simulate the change in salinity expected in coastal waters, by rapidly adding MilliQ water enriched with L1 medium nutrients (23% vol/vol). Control cultures received the same volume of L1 medium at 35 salinity. Salinities were verified with a Vista Refractometer (A366ATC) for accuracy. RNAs were extracted from 45mL of NT and TW cultures at <1hr, 6hr, and 24hr after osmotic shock. Physiological assays were performed using PAM-Fluorometry and a Free Glycerol Assay, and they included the addition of SP1 and TXB4 clonal samples, as well as a 12hr time point to the <1, 6, and 24hr time course setup.

Brevetoxin analysis

Brevetoxin extractions were performed using the ethyl acetate method as described in (Errera *et al.*, 2010).

mRNA extraction and analysis

At each time point, 45 mL NT and TW samples were concentrated by centrifugation (800 × g, 10 min) and RNA was extracted from the pellets in 40 μL of nuclease-free water using the Qiagen RNEasy Mini Kit (Qiagen Inc., Valencia, CA), in accordance with the manufacturer's protocol. Extracted RNA samples were stored at -80°C until library preparation. The RNA concentration and purity of each extraction was estimated by measuring the absorption spectra of 1 μL sample aliquots with a NanoDrop Spectrophotometer and BioAnalyzer RIN score with a threshold of 5 or higher. RNA-Seq data was created from RNA extraction at each time point and for each replicate in both NT and TW clones. Library preparation and Illumina High-Seq sequencing was performed at Michigan State University Research Technology Support Facility (RTSF). RTSF prepared sequencing libraries with the Illumina Stranded mRNA Library Prep Kit LT. Next, 100 bp paired-end short reads were sequenced with the Illumina HiSeq 2500 Rapid Run flow cell (v1), using Illumina Real Time Analysis software v 1.17.21.3 for base calling.

Raw reads were mapped to the *K. brevis* Wilson (TW) reference transcriptome (Ryan *et al.*, 2014) using Salmon-Sailfish 0.7.6, 2015 (Patro *et al.*, 2015). The R program Bioconductor EdgeR was used to identify differentially expressed genes using classic

parameters and a False Discovery Rate (FDR) of <0.05 and a threshold differential expression of 1.5–fold (Chen *et al.*, 2011). Because the *K. brevis* reference transcriptome is not well annotated, unigenes with statistically significant fold changes of 1.5X or greater were subsequently identified via Basic Local Alignment and Search Tool (BLAST) algorithms hosted through the Supercomputing Facility at Texas A&M University (Ryan *et al.*, 2014) and the Marine Microbial Eukaryote Transcriptome Sequencing Project (Keeling *et al.*, 2014), SwissProt, and UniProt gene sequence databases using the nucleotide sequences. Gene identities through these databases were constrained by an E-value <1E-05 for quality of gene identification.

Gene enrichment

NT *vs.* TW differentially expressed genes were clustered into the control and stress samples, then analyzed using the BLAST2GO pipeline of BLASTX alignment, GO mapping function, GO term annotation, and GO gene set enrichment analysis with default parameters and an E-value <1E-05 and FDR rate of 0.05. The reference set of genes were the published reference transcriptome (Ryan *et al.* 2014) and the test set of genes were the differentially expressed genes found by EdgeR for each control and stress in TW *vs.* NT. The number of over-represented genes in each category was then compared to the total number of genes in all categories present for percentages of over-expression by each functional category.

PAM Fluorometry

Photosynthetic efficiencies of the toxin-producing TW and TXB4, no toxin detected NT, and low-toxin producing SP1 clones were compared using pulse-amplitude modulated (PAM) fluorometry (PHTYO-PAM-II, Walz GambH, Effeltrich, Germany) and Phyto-Win 3 Software V 3, 2016. Triplicate control and salinity treatment samples were each dark adapted for 30min, then analyzed using the light curve function with 20 intensities of actinic red light and measuring the Photosynthetic Active Radiation (PAR) *vs.* Electron Transport Rate (ETR) in µmol electrons·m^{-2}·s^{-1}). The Epp model was used to calculate α from each curve, defined as the initial slope of the light-limited region of the curve, considered to be an estimate of the quantum photosynthetic efficiency of the algae in the sample (Kirk, 1994, Eilers & Peeters, 1988). All experiments were repeated twice. Differences between and within clones and treatments were determined using ANOVA 2-way comparison with replication tests.

NPQ values were calculated for TW and NT clones as: $yield = (Fm / Fm') / Fm'$

where Fm is the maximal fluorescence after 0.5 sec actinic red light pulse and Fm' is the maximal fluorescence after 0.5 sec actinic red light pulse of the light adapted sample. These NPQ values were compared between and within clones and treatments using ANOVA 2-way comparison with replication tests.

Photosynthetic yield was calculated for TW, NT, SP1, and TXV4 dark-adapted samples from the following equation: $yield = Fm' – Fo / Fm'$

where Fo is the minimal fluorescence and Fm' is the maximal fluorescence after 0.5 sec

actinic red light pulse. These yield values were compared between and within clones and treatments using ANOVA 2-way comparison with replication tests.

To determine if ROS was an initial stress indicator, we tested the response of NT and TW clones to ROS stress and the subsequent α-values were measured using the PAM Fluorometry methods described above. At <1hr a 0.1µM hydrogen peroxide stress was applied to stress cultures in the same manner as the hypo-osmotic stress experiments, and L1 medium at the same volume was added to control replicate cultures. Measurements by PAM fluorometry were recorded immediately after the stress and at 6hr after stress. The α-values were compared between and within clones and treatments using ANOVA 2-way comparison with replication tests.

Glycerol measurements

Intracellular glycerol assays were performed on replicate control and salinity shift (35→27) stress cultures of TW, NT, TXB4, and SP1 clones of *K. brevis* extracted at time points <1hr, 6hr, 12hr, and 24hr after initial osmotic shock following the same protocol as for the mRNA extractions. Samples from each time point were concentrated by gentle centrifugation (800 × g, 10 min) into pellets of 1.5 X 10^5 cells and the supernatant discarded. To obtain measurements of intracellular glycerol, we used a Colorimetric assay. Sigma-Aldrich Free Glycerol Assay Kit MAK-117 was used with a KC Plate reader BIO-TEK©, Synergy HT to measure absorbance at 570nm to compare for a standard curve of 0-1mM glycerol.

3. RESULTS

Growth rate

In all experimental treatments, NT and TW cultures showed no significant difference in specific growth rate, (0.25 div^{-1}, +/- 0.01). Specific growth rate was lower (0.20 div^{-1}, +/- 0.01) for SP1, and slightly higher for TXB4 (0.26 div^{-1}, +/- 0.02) across experiments.

Toxin Verification

All NT cultures had no detectable brevetoxin or its derivatives in ethyl acetate extracted samples. TW cultures had 8–10 $pg \cdot cell^{-1}$ of brevetoxin, TXB4 cultures had 13–15 $pg \cdot cell^{-1}$, and SP1 cultures had 2–4 $pg \cdot cell^{-1}$.

Differential Gene Expression

Differentially expressed genes found by EdgeR included 1092 unique transcripts. Of those, 220 transcripts were annotated, and further only 103 of the 220 genes were relevant to our osmotic shock experiment.

Toxic vs. NT Wilson Gene enrichment

An enrichment test was used to identify gene categories that were more highly represented among differentially expressed genes in TW *vs.* NT under control and under stress treatments. Gene categories more highly expressed in TW than in NT (Table 1) included nine categories in both control and stress analyses: Small molecule synthesis, Transmembrane transport, Protein folding, Ion binding, Carbohydrate metabolism, Amino acid synthesis, and Precursor metabolite synthesis. Small carbohydrate synthesis and Carbohydrate metabolism categories include genes such as Fructose 1, 6

bisphosphate and Glyceraldehyde-3-Phosphate, which are involved in the Calvin Cycle of normal cell metabolism. Transmembrane transport and Ion binding categories include genes involved in the transport of molecules and ions for normal cell functions. Amino acid synthesis and Precursor metabolite synthesis are categories for genes such as fumarate hydrogenases and cytochrome oxidases that the cell uses to make amino acids and other metabolites to maintain normal processes.

Gene categories that were more highly represented among differentially expressed genes in NT than in TW (Table 2) included six categories in both control and stress analyses: Stress response protein folding, Oxygen evolution, Lipid export, Cytoskeleton maintenance, GTPase activity, and ATPase activity. Stress genes for incorrect protein folding include heat shock proteins (*hsp70, hsp90*) that are highly expressed under stressors that can effect protein formation. Stress genes corresponding to ROS (oxygen enhancers) and GTP/ATPase genes highlight the need for energy molecules to attempt to recover cell homeostasis. Categories also included lipid export (ABCA lipid transport genes) and alpha tubulin genes.

Stress vs. Control

Measuring differential gene expression between control and hypo-osmotic stress replicates at each time point should identify gene transcripts that increased in response to osmotic shock. In response to hypo-osmotic stress, significant differential gene expression in TW control *vs.* stress assays was observed in photosynthetic genes (Figure 1). In particular, PSII genes with the following functions showed 2–7.5-fold lower

expression levels in stressed cultures compared to control cultures: PSII-D&F (core reaction center of PSII), PSII-C (core antenna), PSII-N (PSII stability), PSII-V(O_2 evolution), and PSII-F (plastocyanin docking). NT did not share this expression trend, only one PSII subunit gene was expressed at 4–fold lower level in the stress cultures, and only at the 24hr time point (Figure 2).

Time course experiments

The decrease in PSII gene expression at the <1hr time point led to the analysis of the individual PSII gene transcripts per million at each time point. Tracking the expression of PSII subunit genes across the time points showed that expression levels of these genes of interest were lower in the TW stress treatment at <1hr, then recovered at 6hr and 24hr time points (Figure 3). No significant change in the expression of these genes was seen in the TW control expression measurements (Figure 4). NT showed no expression trend in the PSII subunit genes for either control or stressed cultures (Figures 5, 6).

PAM Fluorometry

Based on the results of gene expression measurements we anticipated that we might observe a decrease in the photosynthetic efficiency in brevetoxin-producing clones. Changes in photosynthetic efficiency were expected based on the differential gene expression assays and to confirm the expected trend in NPQ observed by Cassell *et al.* (2015). PAM measurements were used to directly measure changes in photosynthetic efficiency in response to hypo-osmotic stress. These analyses showed that the NT clone

had a significant deficiency in NPQ compared to the TW clone ($p < 0.05$) under the same conditions (Figure 7).

TW responded immediately to ROS stress with a decrease in photosynthetic efficiency (α value) at <1hr, with a recovery to the control value at 6hr. In contrast, NT showed no significant difference in α in control or ROS-stressed cultures (Figure 8), indicating a possible defect in the response pathway to external ROS stress.

Significantly lower α values were seen immediately (<1hr) in the hypo-osmotically stressed cultures of both toxin producing TW and TXB4 clones; whereas no significant change was observed α for in the low or non-toxin producing SP1 and NT clones following the hypo-osmotic stress treatment (Figures 9, 10).

No significant differences in α values between TW and NT *K. brevis* clones were found, and the only significant differences in photosynthetic quantum yield (ϕ value) were found in the control *vs.* stressed cultures of *K. brevis* clones SP1 (<1hr) and TXB4 (12hr) (data not shown). This was unexpected, as we anticipated NT and SP1 to both have a lower quantum yields due to their low levels of brevetoxin production.

Glycerol

Intracellular glycerol was measured in NT, TW, TXB4, and SP1 clones after hypo-osmotic stress and compared to control cultures to identify changes in the concentration of the small molecule in response to osmotic shock. Toxin-producing TW and TXB4 clones showed a decrease in their intracellular glycerol concentration at the <1hr time point, while the low toxin SP1 and non-detectable toxin NT clones showed no

significant change at <1hr. Time points 6hr, 12hr, and 24hr showed no significant difference in intracellular glycerol concentrations between control or stress cultures for any clone (Figures 11, 12).

4. CONCLUSIONS

Identifying the responses of *Karenia brevis* cells to a rapid salinity shift that can occur as cells move into coastal or estuarine waters is of particular interest for the Gulf of Mexico where blooms can occur annually (Steidinger *et al.*, 1998). Identifying molecular responses behind the response to osmotic stress could be necessary to understand the role of brevetoxin in the cell.

Dinoflagellates can acclimate to osmotic stress using contractile vacuoles, membrane manipulation, and managing metabolites (Kirst, 1990). Commonly, osmoacclimation involves the glycerol molecule via the carbon fixation process in photosynthesis and the degradation of starch (Suescún-Bolívar & Thomé, 2015). Proline is also observed to change in intracellular amount in response to a salinity shock treatment (Delauney & Verma, 1993). Other related metabolites that algae are known for producing in response to osmotic stress include other small carbohydrates such as sorbitol, dulcitol, and dimethlysulfoniopropionate (DMSP) (Boscari *et al.*, 2002, Karsten *et al.*, 1996). Identifiable responses in photosynthetic processes by algae have shown these processes can be under transcriptional control (Frenkel *et al.*, 2009), and that they can be induced by a change in the osmotic balance of their surrounding environment (Dittami *et al.*, 2009). Photosynthetic yield and carbon acquisition was decreased during salt stress in the algae *Chlorella* (Kebeish *et al.*). The hypo-osmotic response however is not the reverse, photosynthetic efficiency of photosystem II also decreases from hypo-osmotic stress (Liu *et al.*, 2012). In red alga *Gracilaria tikvahiae*, hypo-osmotic stress caused a transient decrease in the rate of photosynthesis, where exosmosis of ionic cofactors of

photosynthesis temporarily decreased the photosynthetic efficiency (Lapointe *et al.*, 1984). As shown by the referenced studies, photosynthetic organisms commonly share a response to osmoregulation by influencing photosynthetic pathways to create or destroy photosynthetic compounds as metabolites, or react to ROS that result in a decrease in photosynthetic efficiencies.

ROS can accumulate in response to osmotic shock (Hernandez *et al.*, 1993). The subsequent photosynthetic response is a consequence of increased levels of ROS and utilizing NPQ to regulate photosynthetic pathways to create or destroy photosynthetic compounds as osmolytes for osmoacclimation, which can result in a decrease in photosynthetic efficiencies or yields (Azzabi *et al.*, 2012, Gao *et al.*, 2014). Photosynthesis rates in algae are decreased by hypo-osmotic stress, and small carbohydrate pathways are activated as an osmotic response, where these molecules are used as osmolytes to be produced or destroyed to obtain osmotic balance. In particular, hyperosmotic stress triggers increases in the production of photosynthetically-derived carbohydrates and amino acids as osmolytes and hypo-osmotic stress triggers a decrease in these same compounds (Kirst, 1990).

Because brevetoxin is located in the same compartment as photosynthetic processes, and low-toxin clones of *K. brevis* possess more ROS and a deficiency in NPQ, the physiological responses are indicative of a brevetoxin-related photosynthetic response to osmotic stress (Cassell *et al.*, 2015, Frenkel *et al.*, 2009).

Transcriptomic analyses show a difference in gene expression between variable brevetoxin clones TW and NT. TW and NT gene enrichment analyses showed enzymes in the glycerol/small carbohydrate and amino acid metabolism pathways are more highly expressed in the TW cultures, and stress response such as oxygen evolution and stress response protein folding genes are more highly expressed in NT. These differences indicate that TW is functioning without a significant stress, and that NT is functioning but with a significant stress, with the only observable difference between the clones as the lack in detectable brevetoxin in NT.

Differential gene expression analyses also support a role of brevetoxin level in responses to osmotic stress. TW showed an initial decrease in expression of photosynthetic PSII genes in response to hypo-osmotic stress. The decrease in PSII expression was seen in stressed TW at <1hr, but no response at 6hr, indicating that the response is a temporary acclimation. No decrease in PSII expression was observed at 24hr, indicating it is likely not a diel pattern of gene expression. These genes of interest are the only annotated, genes that are significantly (FDR <0.05) differentially expressed between control and stress treatments of TW. The finding of NT not showing this expression pattern is important as this further implicates the role of brevetoxin in an osmotic, stress generated photosynthetic response. Our transcriptomic results showed that in response to hypo-osmotic stress, toxin producing *K. brevis* alters its gene expression to decrease the photosystem II efficiency, and non-detectable toxin producing clones do not. This suggests that NT is defective in responding to hypo-osmotic stress, further implicating the level of brevetoxin as key in osmotic responses for a *K. brevis* cell.

PAM fluorometry time course results also support a model in which decreased Photosystem II efficiency is a temporary osmotic stress response. The observed decreases in photosynthetic efficiencies in toxin-producing TW and TXB4 cultures, but not in not-detectable/low-toxin producing NT and SP1 cultures, in response to both osmotic and ROS stress suggest that levels of brevetoxin in the cell are linked to amount of ROS. This ROS concentration is subsequently linked to a hypo-osmotic stress response. No consistent changes were observed in photosynthetic yield, suggesting that the decrease in photosynthetic efficiency is not long-lived or severe enough to lead to an overall change in the yield. This further supports the hypothesis that brevetoxin is involved in PSII response to osmotic stress, likely triggered by the increase of intracellular ROS that induces NPQ in order to osmoacclimate. The decrease in photosynthetic efficiency helps support our differential gene expression trend results, as it coincides directly with the decrease in PSII subunits gene expression. Therefore, our NT clone showing no change in the differential gene expression and fluorometric experiments suggests that a non-detectable toxin producing *K. brevis* clone has high production of ROS similar to the findings in Cassell *et al.* (2015). This finding indicates that because osmotic stress involves overproduction of ROS in PSII, a small increase from hypo-osmotic stress would not be a strong enough signal to induce a response. The decrease seen in photosynthetic efficiency of TXB4, and the lack of response by SP1 in osmotic stress experiments, further support this hypothesis.

Hypo-osmotic stress in algae results in the destruction of intracellular glycerol to osmoacclimate the cell (Suescún-Bolívar *et al.*, 2012, Suescún-Bolívar & Thomé, 2015,

Suescún-Bolívar *et al.*, 2016, Ahmad & Hellebust, 1986, Dittami *et al.*, 2009). Our observations of a decrease in intracellular glycerol concentration only in the <1hr of toxic TW and TXB4 clones followed this trend, and are consistent with the differential gene expression and fluorometric results where PSII gene expression and photosynthetic efficiency both respond to stress by a decrease only at the <1hr time point. The glycerol assay results showed the same trend that the low/not-detectable toxin-producing SP1/NT cultures are affected from lack of brevetoxin and cannot react to the additional osmotic stress.

In summary, differential gene expression analysis showed a definite initial trend of a decrease in photosystem II gene expression in response to hypo-osmotic stress in TW, and no such trend in NT. These results are supported by physiological assays from PAM flourometry and intracellular glycerol assays, with responses seen in normal toxin-producing clones only. These results support our hypotheses that NT is defective in response to osmotic shock as compared to TW, and that less than normal brevetoxin production prevents a response in *K. brevis* clones. The support of these hypotheses suggests the level of brevetoxin production is linked to the photosynthetic stress response of *K. brevis* cells, and further that the level of brevetoxin production is key to a hypo-osmotic stress response in *K. brevis* clones. Our results also support the ROS induction of NPQ in the case of hypo-osmotic stress in *K. brevis* for the purpose of osmoacclimation in the chloroplast, where adequate brevetoxin production is needed to effectively achieve NPQ. Further work in this field could help identify the definitive function of brevetoxin in the photosynthetic processes of *K. brevis*.

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APPENDIX A

TABLES

TW vs. NT Enrichment Analysis, Control Cultures	Percent Genes with Annotated Function
Genes expressed more in TW	
Oxidoreductase, small molecule metabolism	13%
Transmembrane transport	6%
Protein folding	19%
Ion binding	6%
Carbohydrate metabolism	19%
Protein translation	13%
Amino acid synthesis	19%
Precursor metabolite synthesis	13%
Genes expressed more in NT	
Stress response protein folding	29%
Oxygen evolution	14%
Lipid export	14%
Cytoskeleten maintenance	14%
GTPase activity	14%
ATPase activity	14%

Table 1. Enrichment analysis of differentially expressed genes in K. brevis TW vs. NT control cultures.

TW vs. NT Enrichment Analysis, Stress Cultures	Percent Genes with Annotated Function
Genes expressed more in TW	
Oxidoreductase, small molecule metabolism	12%
Transmembrane transport	6%
Protein folding	18%
Ion binding	6%
Carbohydrate metabolism	12%
Protein translation	12%
Amino acid synthesis	12%
Precursor metabolite synthesis	24%
Genes expressed more in NT	
Stress response protein folding	25%
Oxygen evolution	13%
Lipid export	13%
Cytoskeleten maintenance	25%
GTPase activity	13%
ATPase activity	13%

Table 2. Enrichment analysis of differentially expressed genes in K. brevis TW vs. NT osmotic stress cultures.

APPENDIX B

FIGURES

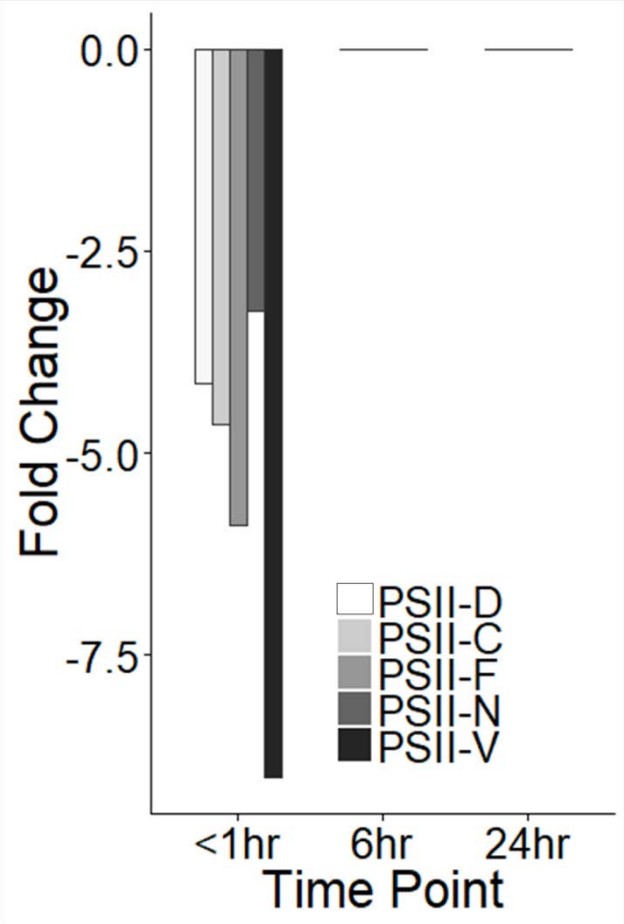

Figure 1. Photosystem II subunit gene expression in the K. brevis TW stress cultures.

A decreased expression of PSII subunit genes was observed at 0hr response to stress, no change was observed at 6hr or 24hr time

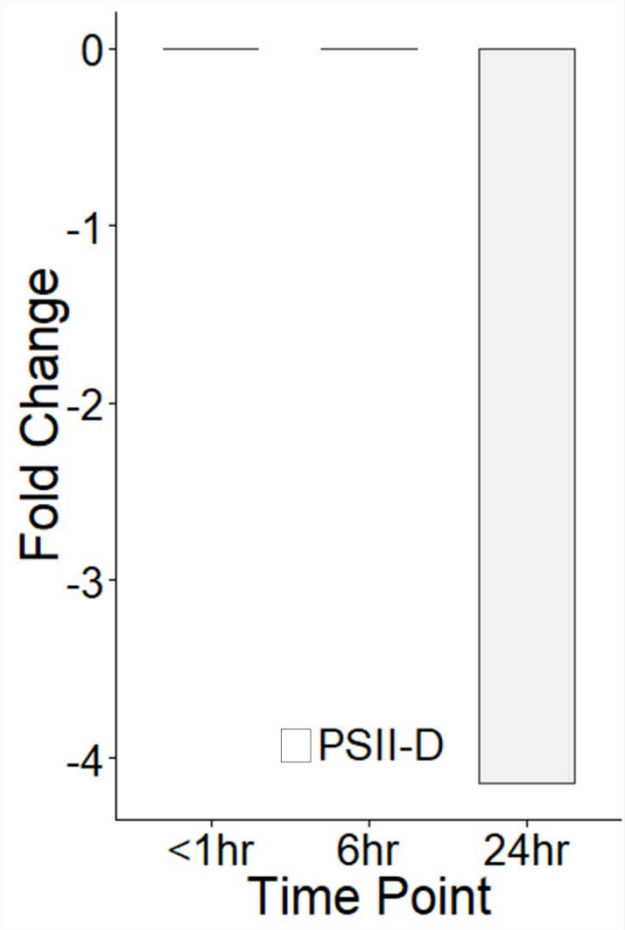

Figure 2. Photosystem II subunit gene expression in the K. brevis NT stress cultures.

No change of expression of PSII subunit genes was observed at 0hr response to stress, no change was observed at 6hr or 24hr time points.

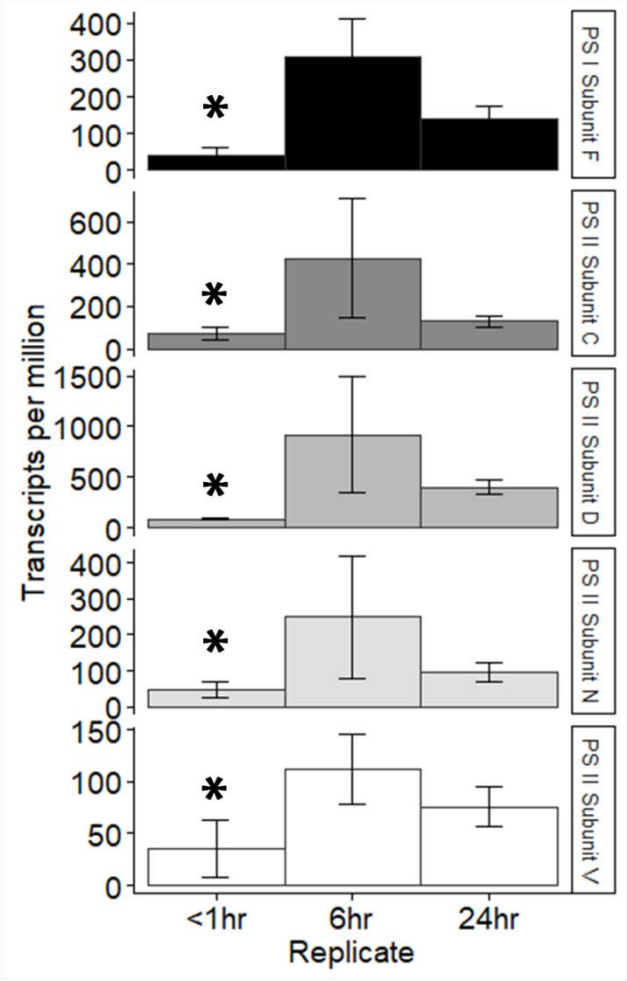

Figure 3. Photosystem II subunit gene transcripts per million in the K. brevis TW stress cultures.

Photosystem I & II subunit gene expression showed significant fold change trend differences over the 0-24hr time points in stress cultures of *K. brevis* TW.

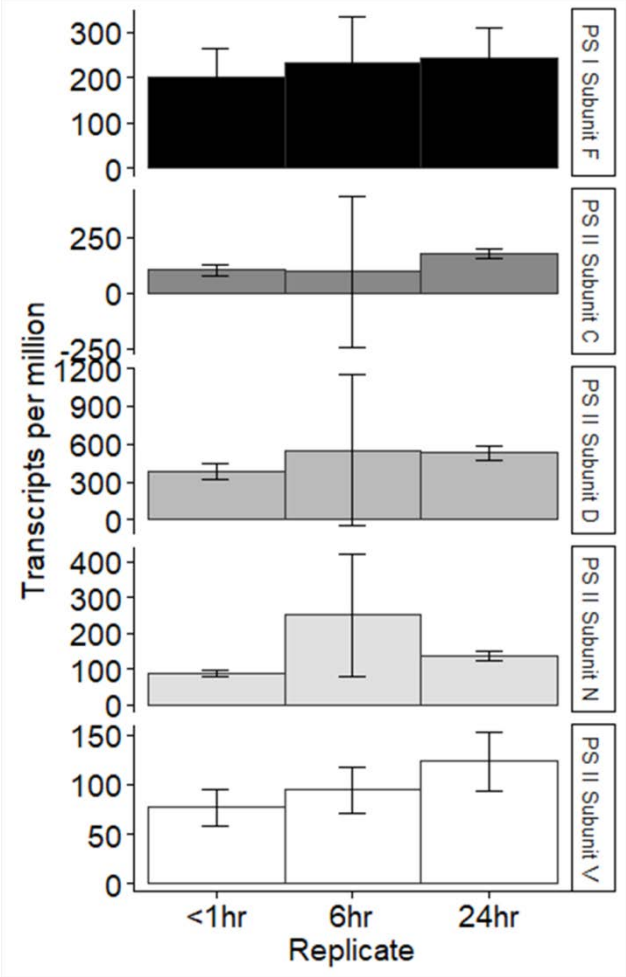

Figure 4. Photosystem II subunit gene transcripts per million in the K. brevis TW control cultures.

Photosystem I & II subunit gene expression showed no significant fold change differences over the 0-24hr time points in control cultures of *K. brevis* TW.

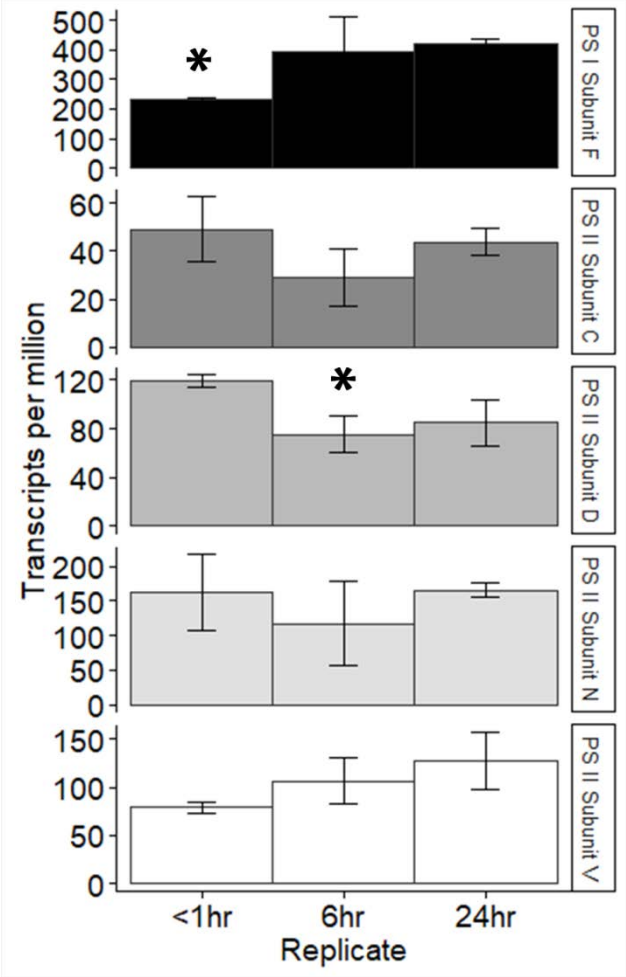

Figure 5. Photosystem II subunit gene transcripts per million in the K. brevis NT control cultures.

Photosystem I & II subunit gene expression showed no significant fold change trend differences over the 0-24hr time points in control cultures of *K. brevis* NT.

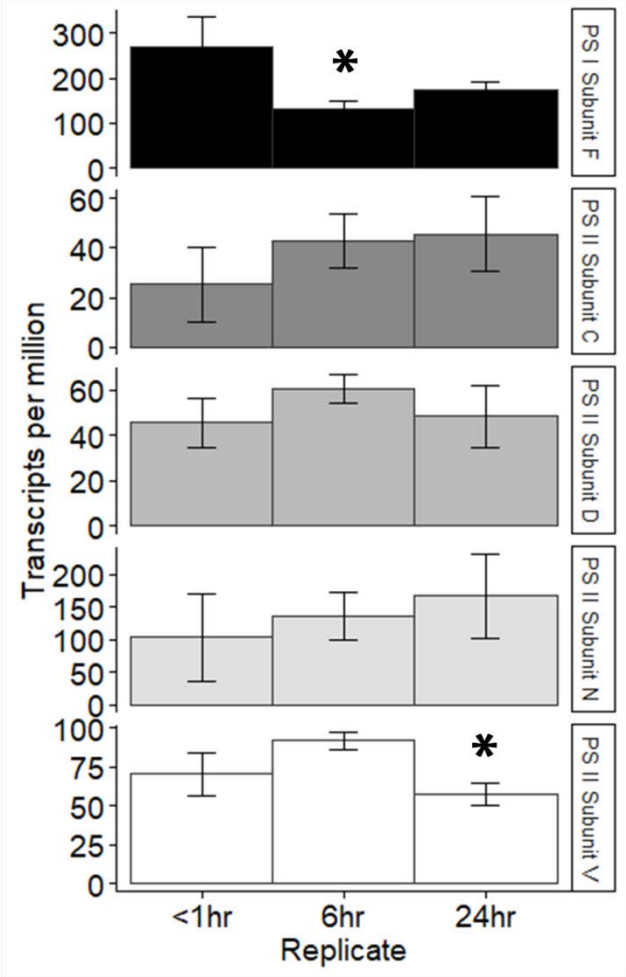

Figure 6. Photosystem II subunit gene transcripts per million in the K. brevis NT stress cultures.

Photosystem I & II subunit gene expression showed no significant fold change trend differences over the 0-24hr time points in stress cultures of *K. brevis* NT.

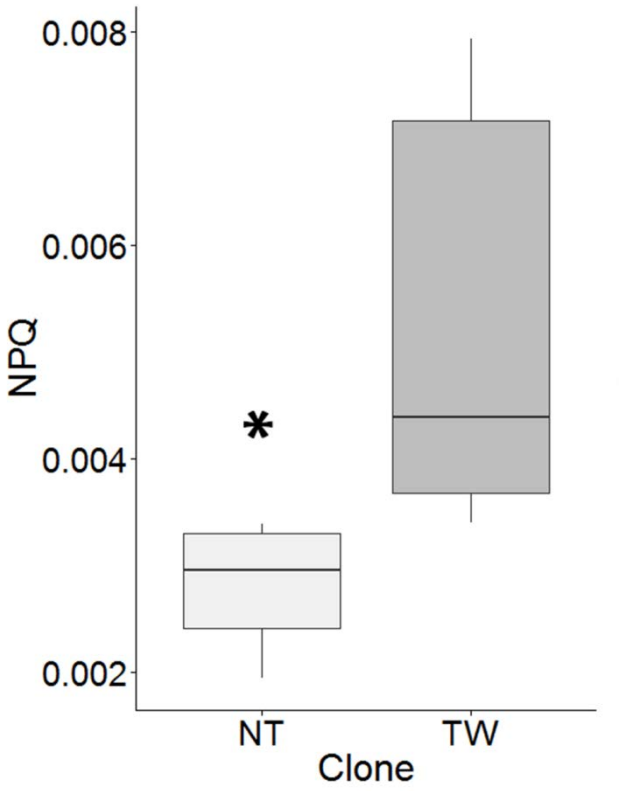

Figure 7. NPQ values in K. brevis NT and TW clones.

NT clone showed a significant deficiency in NPQ as compared to the TW clone.

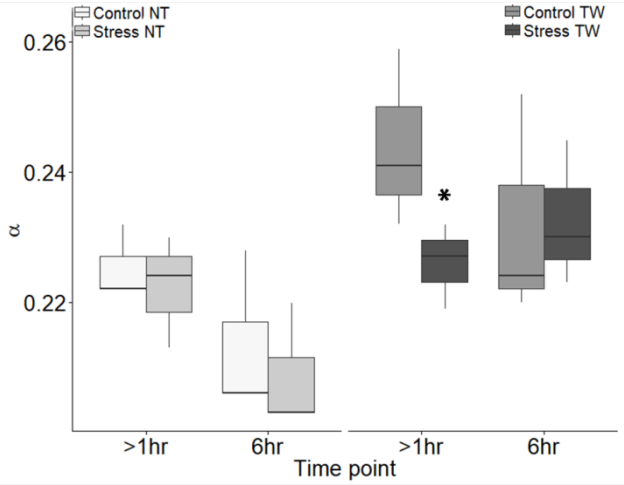

Figure 8. K. brevis TW and NT replicates of control and ROS shifted culture (0.1µM H_2O_2) at 0hr and 6hr time points after initial stress.

At 0hr time point, a significantly lower α value was observed by the stressed sample mean. 6hr time point and comparison did not show a significant α value difference between control and stress.

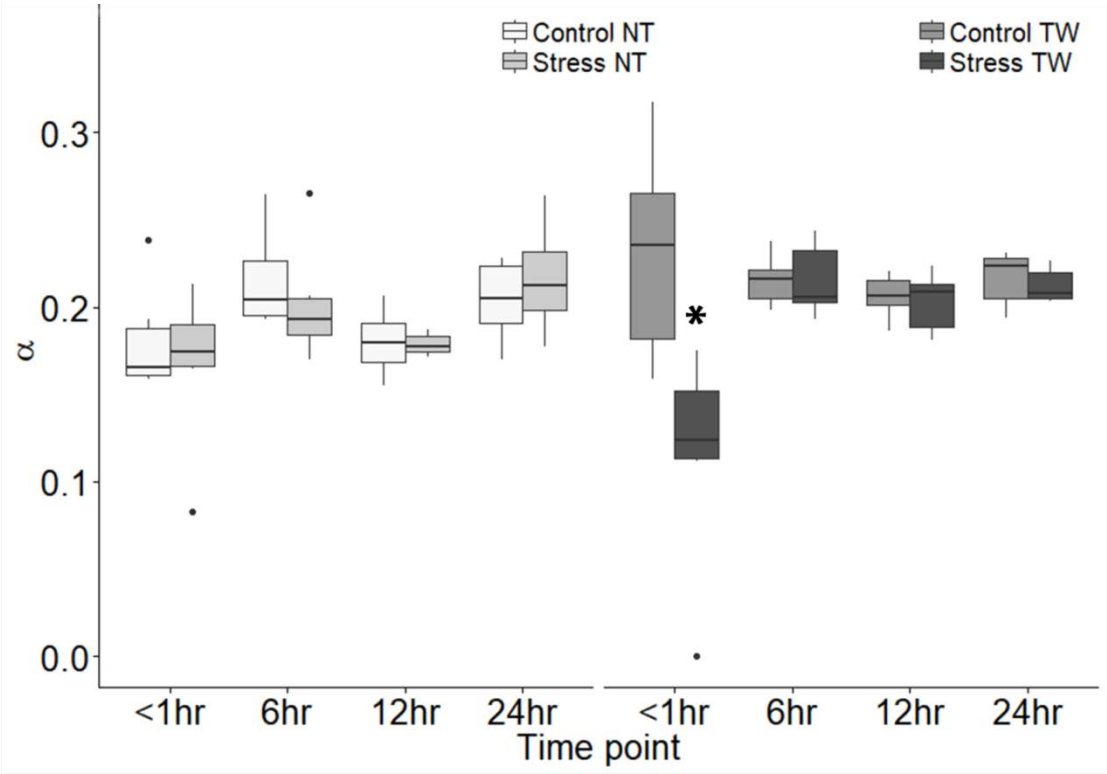

Figure 9. Boxplot of mean α values in K. brevis hypo-osmotic stress PAM Fluorometry experiments, TW and NT.

TW and NT replicates of control and salinity shifted (35ppt→27ppt) culture at 0hr, 6hr, 12hr, and 24hr time points after osmotic shock. At 0hr time point, a significantly lower α value was observed by the TW stressed sample mean. All other time points and comparisons did not show a significant α value difference between control and stress.

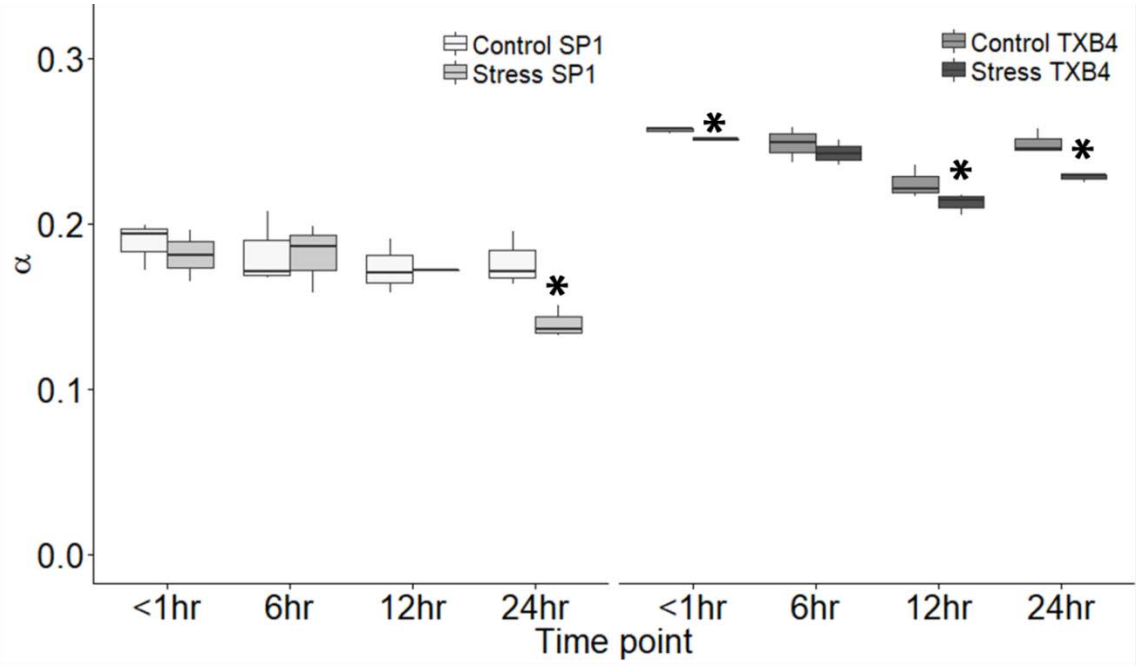

Figure 10. Boxplot of mean α values in K. brevis hypo-osmotic stress PAM Fluorometry experiments, TXB4 and SP1.

TXB4 *K. brevis* clone and SP1 *K. brevis* clone replicates of control and salinity shifted (35ppt→27ppt) culture at 0hr, 6hr, 12hr, and 24hr time points after initial osmotic shock. At 0hr, 12hr, and 24hr time points, a significantly lower α value was observed by the TXB4 stress sample mean. All other time points and comparisons did not show a significant α value difference between control and stress with the exception of SP1 24hr.

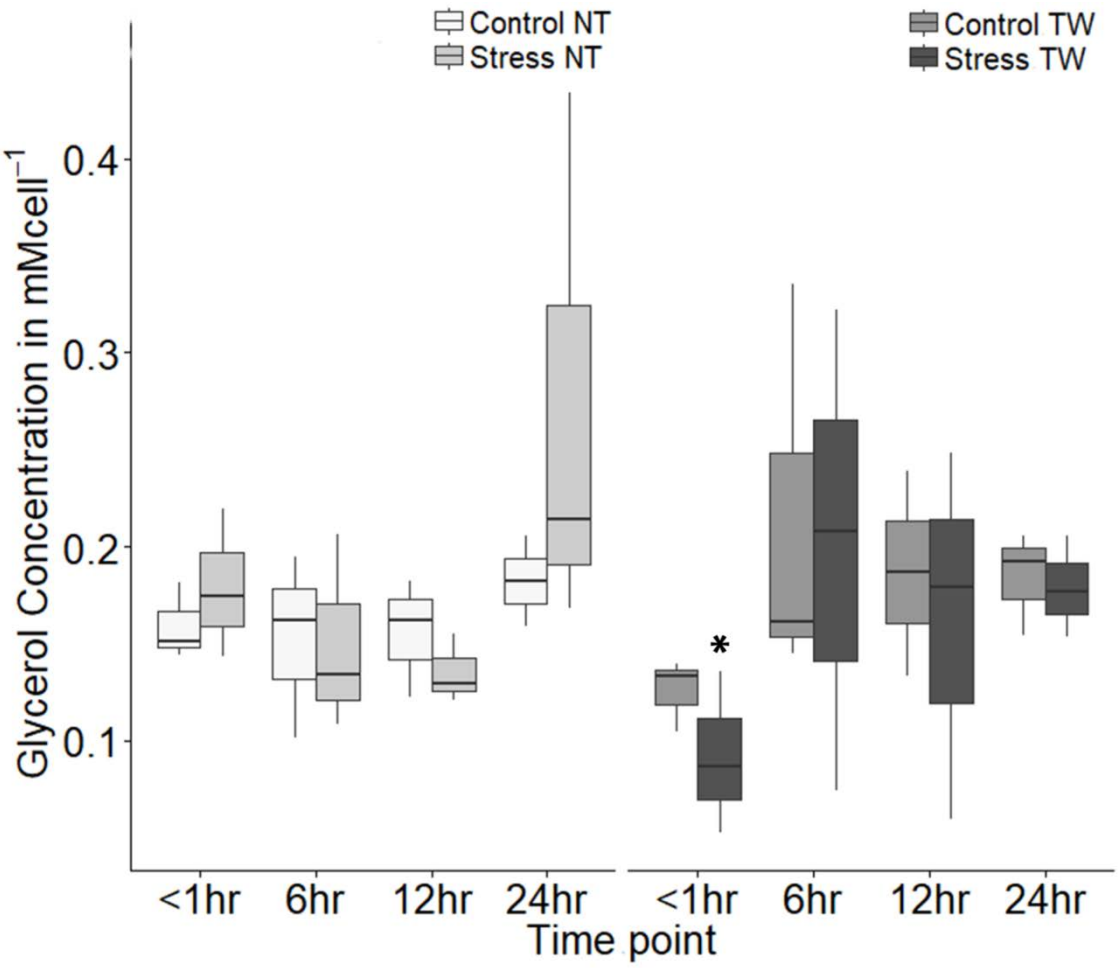

Figure 11. Glycerol concentrations of K. brevis TW and NT.

Replicates of *K. brevis* clones TW and NT control and salinity shifted (35ppt→27ppt) culture at 0hr, 6hr, 12hr, and 24hr time points after initial osmotic shock. A significant decrease in intracellular glycerol concentration was observed in toxic clone TW, and no significant decrease was observed in non-detectable toxin clone NT. Concentrations were standardized to 1.5^5 cells.

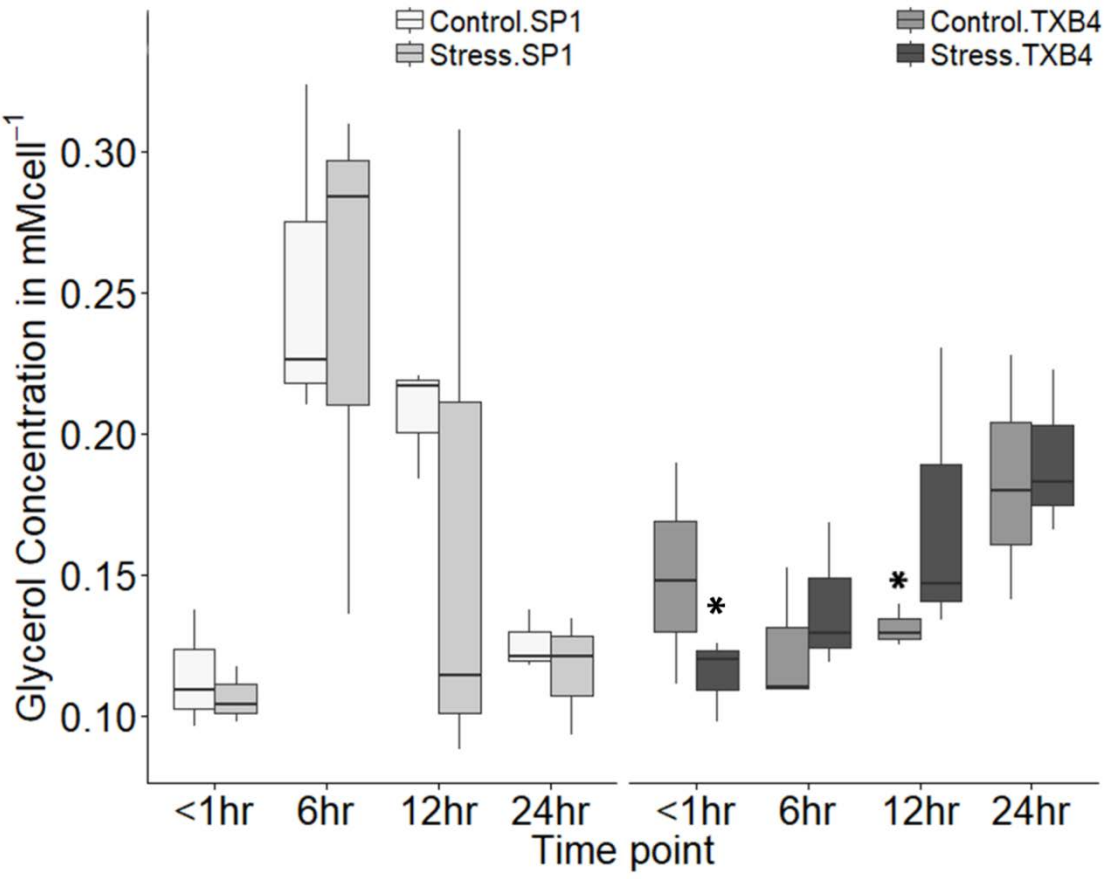

Figure 12. Glycerol concentrations of K. brevis SP1 and TXB4.

Replicates of *K. brevis* clones TXB4 and SP1control and salinity shifted (35ppt→27ppt) culture at 0hr, 6hr, 12hr, and 24hr time points after initial osmotic shock. A significant decrease was observed in toxic clone TXB4, and no significant decrease was observed in low-toxic clone SP1. Concentrations were standardized to 1.5^5 cells.