Transcriptome-based expression analysis and O_2 generation of algae growing under Mars-relevant low pressure and variable light intensities and wavelengths.

L.M. Cycil[1], E.M. Hausrath[1], J. Raymond[2], D.W. Ming[3], C. Adcock[1], W.P. Ruemmele[3], J. Petereit[4], H.A. Vasquez-Gross[4] [1]UNLV, Dept. of Geoscience, Las Vegas, NV, USA, [2]UNLV, School of Life Sciences, Las Vegas, NV, USA. [3]NASA JSC, Houston, TX, USA.[4]Nevada Bioinformatics Center (RRID:SCR_017802), Reno, NV USA

Introduction: Human exploration of environments beyond Earth's atmosphere has brought attention to unique challenges faced by photosynthetic organism such as algae proposed to be used as components of bioregenerative life support system (BLSS) for food and O_2 production [9]. Our previous studies showed that candidate algae species, *Chloromonas brevispina, Dunaliella salina,* and *Chlorella vulgaris* showed substantial growth at pressures as low as 80 mbar [3] and low light intensities relevant to Mars[2], which are about half those on Earth.

Here, we examine the gene expression profiles of the extremophilic algae *C. brevispina* and *D. salina* growing under low pressure (160 mbar CO_2) as compared to the controls growing under Earth's atmosphere conditions (1013 mbar terrestrial atmosphere). We also quantified the oxygen generation and biomass production of the algae species growing under combined stressors of low pressure (160 mbar), low light intensity (500μmol $m^{-2}s^{-1}$), and different light wavelengths including red, blue, and full spectrum white light.

Methods: *Algae growth conditions.* The algae species *C. vulgaris, C. brevispina,* and *D. salina* were cultured in triplicate in a 3 Gallon Aluminum vacuum chamber (SlickVacSeal) with a clear tempered glass lid in a CO_2 atmosphere at a pressure of 160 mbar and 500 μmol m^{-2} s^{-1} light intensity under red (620-750nm), blue (450-495nm), and full spectrum white light [3]. Sampling was performed once a week until cultures reach their carrying capacity, and the atmosphere was evacuated and purged with CO_2 three times after every sampling. Extremophilic species *C. brevispina* and *D. salina* grown as described above, but under full spectrum white light (62–70 μmol m^{-2} s^{-1}) after [3,5], andwere further analyzed for differential gene expression as described below.

RNA Extraction and Purification. RNA was extracted from the algae cells where the cell pellets were first lysed mechanically using liquid nitrogen and then using a Trizol reagent [4]. RNA was purified using the ZR *RNA MiniPrep (Zymo)*. The quantity, purity, and integrity of each sample were determined using the Qubit Assay (ThermoFisher), NanoDrop (ThermoFisher), and Bioanalyzer (Agilent), respectively. RNA sequencing was performed using Illumina NextSeq 500 (Illumina) for 6 samples of both *C. brevispina* and *D. salina* algae species including 3 replicates of algae growing at Earth's atmospheric pressure (1013 mbar) and 3 replicates of algae growing at low-pressure conditions (160 mbar).

De novo Transcriptome Assembly and Gene Expression Analysis. The raw sequencing data (fastq files) were trimmed and filtered using FastQC. Pooled filtered reads were used for the *de novo* assembly for each species using Trinity v2.85. The completeness of each reference transcriptome was assessed using Benchmarking Universal Single-Copy Orthologs (BUSCO) v5.2.2 [8] and the Chlorophyta odb10 dataset. The reference transcriptomes were annotated with EnTAP v0.10.8 and EggnogDB v5. The libraries were individually aligned to their respective reference transcriptome using HISAT2 v2.2.1, and transcript counts were generated using FeatureCounts from the subread package v2.0 [7]. Reads were then imported into R v4.2.1, contigs with too few reads or extreme outlying characteristics were removed, and differential analysis was conducted with the DESeq2 v1.36.0 pipeline. Statistical differential expression was defined by an absolute log2 Fold Change of > 1 and an false discovery corrected *p*-value of < 0.05. Principal Component Analysis (PCA) was used to verify treatment effect.

Biomass and O_2 generation. Algal biomass was measured as previously described [3] by measuring optical density (OD) using a UV/Vis spectrophotometer (GENESYS) and cell counting using disposable hemocytometer chambers (Incyto CChip, Neubauer) under 400× magnification on an Olympus BH microscope. A 0.5 ml subsample of the gas headspace was collected weekly through a septa sealed valve on the low pressure chamber using a gas syringe (Hamilton), and then injected into an SRI 8610C gas chromatograph (GC) with a Hayesep D column [1]. Sampling and injection procedures were performed consistently to minimize both O_2 introduction (e.g. from needle or mechanical valve dead spaces) and impact on relative O_2 results. Calibrations were done using 1, 5, 10, 15 and 20% certified reference gas standards (Airgas). The O_2 standards were run with the samples each time to quantify the O_2 concentration and were prepared in sterile serum vials. Serum vials were N_2 purged in an N_2 glove box and then capped and crimped with Septa. These capped bottles were flushed with each O_2 standard four times for 5 minutes with 5 minute intervals between each flush. O_2 peaks generat-

ed for samples and standards were further processed using OriginPro (9.9.0.220) and Microsoft Excel.

Results and Discussion:

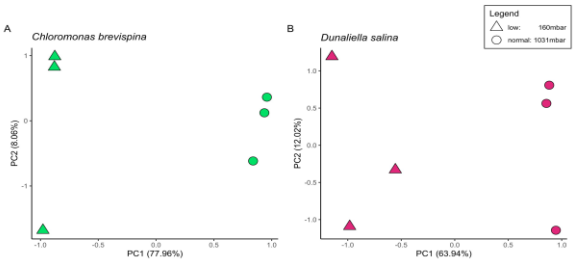

Figure 1. *Validation of treatment effect on gene signatures by principal component analysis (PCA) of two extremophilic algae C. brevispina and D. salina exposed to low pressure conditions of 160 mbar CO_2 and Earth's atmospheric pressure of 1031 mbar; first principal component (PC1) present variance caused by treatment and second principal component (PC2) demonstrate intra-replicate variance.*

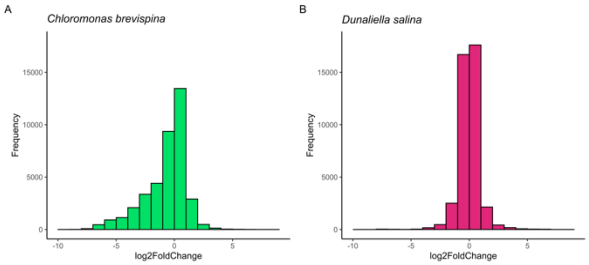

Figure 2. *Distribution of log2 Fold Changes obtain from the differential expression analysis of algae species A) Chloromonas brevispina and B) Dunaliella salina under low pressure condition (160 mbar) as compared to Earth's atmospheric pressure (1031 mbar).*

Gene Expression Analysis. The differential gene expression analysis of two extremophilic algae species *C. brevispina* and *D. salina* indicate clear differences between the transcriptome profiles of algae growing under the two different pressure conditions. PCA showed about 77.9% and 63.9% variability and distinct clustering between treatment (160 mbar CO_2) and control (1031 mbar atmospheric pressure) groups in *C brevispina* and *D. salina*, respectively (Figure 1). Figure 2 demonstrates the log2 Fold Change distribution indicating larger transcriptional changes in *C. brevispina*. The fact that *D. salina* was able to grow better while expressing many fewer genes indicates that *D. salina* may have already established genetic mechanisms to survive multiple stresses. Work is currently ongoing to better annotate and classify genes that are up- and down-regulated by low pressure/high CO_2 compared to Earth's atmospheric pressure conditios for each species, *C. brevispina* and *D. salina*.

Biomass and O_2 generation. Overall, the preliminary results indicate that higher percent concentration of O_2 production was observed under white light for all species (Figure 3).

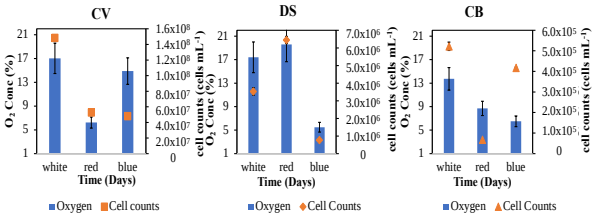

Figure 3. *Maximum O_2 and associated cell counts observed under white, red and blue light at a pressure of 160 mbar CO_2 and 500 µmol m^{-2} s^{-1} of light intensity for, C vulgaris (CV), D. salina (DS) and C. brevispina (CB). The analytical error of the O_2 concentrations was estimated as ± 15%. Error bars on the cell counts are the standard deviation of the mean cell count values. Where error bars are not seen they lie within the points.*

Conclusion: Exposure to Mars-relevant reduced pressure is a novel abiotic stress that is outside the evolutionary experience of terrestrial photosynthetic organisms, and little is known about the adaptive mechanisms and patterns of gene expression of algae under low atmospheric pressure [9]. The gene expression analysis showed a clear difference between the transcriptome profiles of species growing under low pressure as compared to the Earth's atmospheric pressure. The differentially expressed genes patterns in response to low pressure may help better develop BLSS able to support human exploration of Mars and other locations. In addition, the substantial O_2 production and biomass generation under the combined stressors of reduced pressure and low light conditions relevant to Mars make these algae species excellent candidates for bioregenerative life support systems.

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