UNIVERSITY OF CALIFORNIA SAN DIEGO

Upregulated Osmoregulatory Mechanisms Improve Salt Tolerance of *Dunaliella* in Co-Culture with Symbiotic Haloarchaea

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Oceanography

by

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Professor Lihini Aluwihare
Professor Douglas Bartlett

2024

The Thesis of Ruth Varner is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego
2024

DEDICATION

To Mom, Dad, and My Daughter, Millie, I would not be where I am today without you

In loving memory of Pumpkin Varner

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ACKNOWLEDGEMENTS

First, I would like to thank my advisor, Professor Jeff Bowman, for giving me the opportunity to expand my knowledge and dive into the world of microalgae. Without his support, I would not have accomplished this feat.

Next, a huge expression of gratitude and thanks to Ph.D. candidate, Benjamin Klempay for his unwavering guidance and support. Regardless of the question or concern, Benjamin always made himself available to assist me, helping me maintain my footing throughout this process. He provided me with the tools to be confident and successful in my research moving forward.

I would also like to acknowledge Professor Malak Tfaily for her extensive support throughout this project. Without her willingness to process the samples with her lab's FTICR-MS this project would not have been possible. Her expertise in this field has also come as a great help to me with interpreting the data and identifying metabolites. Her invaluable assistance was an enormous help in this process.

Moreover, I would like to offer my deepest thanks to Melissa Hopkins for all the work she conducted on the project prior to my start at Scripps. It is due to her initial legwork in collecting the samples and her initial design of the co-culture experiment that I was able to complete this project in just two years. Her contribution is invaluable and deeply appreciated.

Lastly, Thank you to the rest of my committee, Professor Douglas Bartlett and Professor Lihini Aluwihare for all your excellent feedback and assistance with this work

ABSTRACT OF THE THESIS

Upregulated Osmoregulatory Mechanisms Improve Salt Tolerance of *Dunaliella* in Co-Culture with Symbiotic Haloarchaea

by

Ruth Varner

Master of Science in Oceanography

University of California San Diego, 2024

Professor Jeff Bowman, Chair

Known for its ecological importance as the most halophilic eukaryotic phototroph, *Dunaliella* is utilized for its salt tolerance in a variety of industrial applications. With rising temperatures and elevated salinity levels, *Dunaliella* has witnessed increased rates of mortality in the process of cultivation. To improve upon current growth conditions, this paper along with recent research has turned toward investigating algal-bacterial relationships. Through co-culture experiments in varying salinities and FT-ICR MS analysis, this paper aimed to understand the

ecological associations between *Dunaliella sp.* 15-1 and eight different heterotrophic prokaryotes. The findings indicated significant enhancement in growth rates, especially at higher salinities for both the *Halolamina* (isolate 025) and *Halorubrum* (isolate 027) species co-cultured with *Dunaliella.* Further investigation of these two co-cultures through metabolomic analysis revealed an upregulation of biological pathways involved in membrane fluidity, osmoregulation through increased compatible solute concentrations and improved ion transport. These pathways are likely upregulated by the presence of heterotrophic prokaryotes and contribute to the improved growth and salinity tolerance of the microalgae, suggesting a mutualistic or commensalistic symbiotic relationship.

INTRODUCTION

Microalgae are unicellular microorganisms renowned for their ability to fix carbon through oxygenic photosynthesis (Hu, Qui, Zhao, and Chen 2018). Spanning a diverse range of both prokaryotic and eukaryotic organisms (Metting Jr. 1996), microalgae have garnered significant attention in various industries. Eukaryotic microalgae, in particular, are highly valued in the biofuels industry for their high lipid content (~70%), and substantial biomass making them an excellent alternative to fossil fuels and non-food feedstock. Their key nutrient-recycling abilities and adaptive plasticity have not only led to major reductions in the consumption of fertilizers and potable water but have also resulted in 15-300 times the oil yield for biofuel production (Ishika, Moheimani, and Bahri 2017; Hussian 2018). Furthermore, their cost-effective growth and prolific production of macromolecules, vitamins, antibiotics, and antioxidants position them as an adept bioreactor system in the creation of recombinant proteins for pharmaceutical purposes (Yan, Fan, Chen, and Hu 2016).

However, despite these numerous benefits, microalgae encounter significant challenges in both pharmaceutical and biofuel applications. In biofuels, a major issue stems from limitations in salt-tolerance. The utilization of open pond systems for large-scale algal cultivation exacerbates this problem, leading to reduced algal growth rates and increased mortality as salinity rises due to seawater use and elevated solar irradiation (Ishika, Bahri, Laird, and Moheimani 2018). Ishika et al. (2018) demonstrates that based on the location and level of irradiance, a cultivation pond could increase salinity from 35 ppt to salt saturation in as little as a year. Moreover, in the case of pharmaceuticals, the recurrent use of pharmaceutical compounds produced from microalgae has resulted in pathogens evolving resistance to these compounds, thereby reducing their effectiveness.

For this reason, in recent years pharmaceutical companies have moved toward exploring novel habitats for new antimicrobial compounds.

Despite these challenges, the green algal genus *Dunaliella* stands out for its remarkable ability to thrive in high-salinity environments and offers promising solutions to these obstacles. Originally discovered in 1838, *Dunaliella* has been identified worldwide in a variety of salt lakes and saltern evaporation and crystallization ponds ranging in temperature, pH, and light availability (Oren 2005). Known for its ability to thrive in a range of sodium chloride concentrations (35 ppt - 300 ppt), *Dunaliella* is the most halophilic photoautotroph known in the world (Oren 2005). Its highly adaptive nature in addition to its abundance and ubiquitous nature in hypersaline environments, fast growth rates, and ease of cultivation has made it an apt model organism for studies involving salt adaptation and osmotic stress tolerance (Oren 2005). Even though *Dunaliella* is a keystone species in hypersaline habitats and provides much of the organic carbon required by its surrounding heterotrophic organisms (Oren 2014), recent research has focused mainly on single-species mechanisms to explain its salt-tolerant nature including osmoregulation through the production and degradation of carotene and glycerol (Oren 2014). However, to fully understand and accurately identify the mechanisms at work, interspecies interactions should be considered. This is supported by previous research finding that certain molecules were only produced or significantly in abundance when organisms of interest were co-cultured with other microorganisms (Marmann et al. 2014). In addition, several studies have also demonstrated impacts on metabolism and biomass accumulation associated with the co-culturing of microorganisms (Le Chevantona et al. 2013; Segev et al. 2016; Van Tol, Amin, and Armbrust 2017).

Algae and other phytoplankton have especially been known to display similar effects to metabolism and the production of certain compounds from co-cultures with prokaryotic

microorganisms. Microorganisms that coexist with these photosynthetic organisms in their natural habitats have been shown to provide a wealth of benefits to their eukaryotic counterparts by providing B_{12} (Cruz-López & Maske 2016; Kazamia et al. 2012), trace metals (Amin et al. 2009; Gutierrez, Biller, Shimmield, and Green 2012), phytohormones like auxin (Ramanan et al. 2016; Tandon and Jin 2017), a reduction in reactive oxygen species (Seymour, Amin, Raina, and Stocker 2017), protective antibiotic compounds (Fuentes et al., 2016; Ramanan et al. 2016; Stock et al. 2019), and/or important macronutrients (Fuentes et al. 2016; Le Chevantona et al. 2013; Ramanan et al. 2016). Due to these beneficial properties, co-culturing of algae with heterotrophic prokaryotes can result in an increase in algal growth and biomass. However, not all effects of heterotrophic prokaryotes on algae are positive. When the two are competing for resources, some heterotrophic prokaryotes can release compounds with algicidal properties (Ramanan et al. 2016; Wang et al. 2018; Wang, Tomasch, Jarek, and Wagner-Döbler 2014). In other cases, relationships that start off mutualistic, can become parasitic with changes in environmental conditions (Grossart and Simon 2007; Le Chevantona et al. 2013; Ramanan et al. 2016). For this reason, it is important to assess all relationships between microorganisms that typically reside in the same habitat as the algae of interest.

Metabolomics is a robust method to assess these relationships as it allows the investigator to see small molecule expressions on a larger scale. Frequently used for the study of microalgae, metabolomics allows researchers to gain insight into cellular responses to environmental stress (Willamme et al. 2015; Sun, Yang, and Wawrik 2018). The observed expression of these molecules can then be used to deduce the nature and function of microalgae's ecological relationship with heterotrophic prokaryotes (Croft et al. 2005; Amin et al. 2015; Seyedsayamdost et al. 2011). When examining natural organic matter, it can be considerably difficult to identify

and analyze each individual component. For this reason, to resolve the compounds of importance, substance-specific, molecular-level mass spectra are required (Ayala-Ortiz et al. 2023). With precision formula assignment, high mass accuracy, and robust, reproducible analyses, Fourier-Transform ion cyclotron resonance (FT-ICR) mass spectrometry is regarded as one of the best approaches for the study of small organic molecules (Ayala-Ortiz et al. 2023).

One specific approach of FT-ICR MS is direct injection mass spectrometry. In this approach the liquid samples are directly added to the mass spectrometer without fractionation. This reduces the analysis time and significantly speeds up sample processing (Ayala-Ortiz et al. 2023). While there are some drawbacks to direct-injection including lack of fine resolving power and inability to separate chemical isomers, this approach provides a detailed molecular profile of the sampled natural organic matter and a notion of how organisms may respond chemically to different factors (Ayala-Ortiz et al. 2023).

In this thesis I applied direct-injection FT-ICR MS following *Dunaliella sp.* grown in co-culture with various halophilic prokaryotes in different salinity conditions to explore the ecological relationships between *Dunaliella* and its prokaryotic counterparts in the context of salt stress, and to better understand the mechanisms facilitating these relationships. I hypothesized that at higher salinities *Dunaliella,* and its prokaryotic counterparts would form a mutualistic relationship as evidenced by enhanced *Dunaliella* growth. Furthermore, I expected to see increased abundance of metabolites involved in processes of osmoregulation and molecules associated with membrane fluidity.

MATERIALS AND METHODS

2.1 Sample collection

The algal and prokaryotic cultures used in this study were isolated from environmental samples collected from South Bay Salt Works (SBSW) in Chula Vista, California on October 6, 2017, and August 7, 2019. Samples included brine collected from the shallow water column (~30 cm) of the evaporation pond by means of an extension pole (Sites 0 and 6), and sediment collected by scraping the edge of the bank with a metal spatula (Sites 6 and 8) (Fig 1). Biochemical parameters for each site were measured in situ with an AQUALAB 4TE dew point water activity meter (A_w), a Hanna HI98194 multiparameter meter (temperature, pH, conductivity, redox), an AquaFlash Handheld Active Fluorometer (chlorophyll), and a Vollrath 318433 Refractometer (salinity) (Table 1.). Water activity and multi-parameter meter measurements were only collected during sampling conducted in 2019.

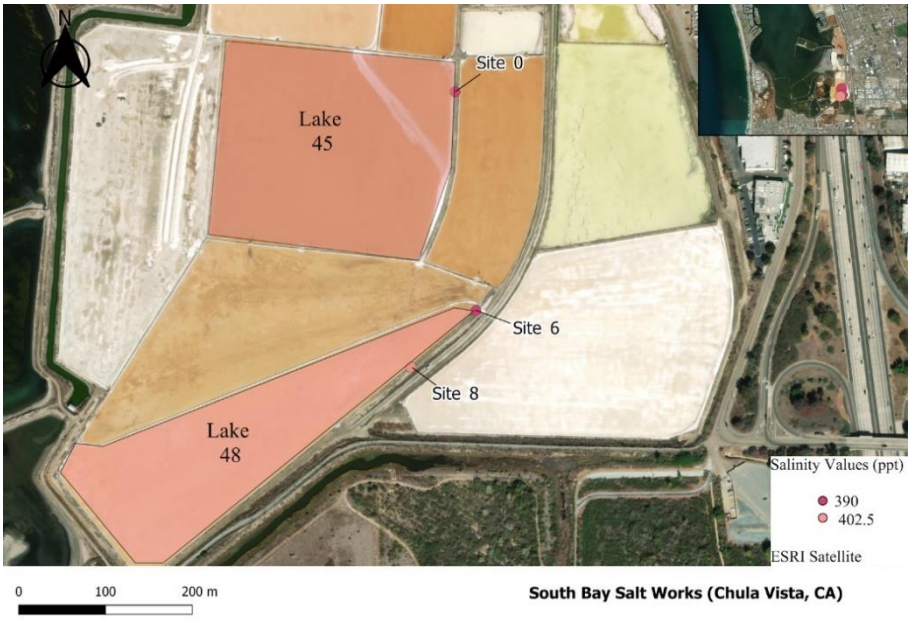

Figure 1. Sample collection sites at SBSW. Sites are color coded by salinity (ppt), and their corresponding lakes are highlighted and labeled. Satellite image of SBSW is provided by Environmental Systems Research Institute (ESRI) GIS mapping software.

Table 1. In situ site measurements

Site	Latitude	Longitude	Lake #	Chlorophyll	Water Activity	Salinity
0	32.5986	-117.09399	45	3.88	n/a	390 ppt
6	32.5963	-117.09373	48	8.97	0.6819	390 ppt
8	32.5958	-117.09453	48	8.92	0.6760	403 ppt

Salinity was determined by diluting samples by a factor of 5 (Sites 6 and 8) and 2 (Site 0).

2.2 Strain isolation

Strains were isolated by inoculating L1 media amended to 121 ppt (Site 0) and 23% Modified Growth Media (23% MGM) (Dyall-Smith 2008,14) (Sites 6 and 8) with collected brine and sediment samples. Autotrophic (L1-121 ppt) enrichments were then incubated at 20^{o}C under a 12-hour light/12-hour dark cycle, while heterotrophic (23% MGM) enrichments were incubated at 37^{o}C under no artificial light. After 2 weeks, active cultures were identified based on turbidity and subsequently streaked three times on agar plates of the same media to isolate individual clonal populations. Morphologically distinct colonies were collected and suspended in the appropriate liquid media.

2.3 Strain identification

Following incubation, an aliquot of each pure culture was collected for strain identification. Autotrophic strains grown in L1-121ppt media were identified by centrifuging an aliquot of pure culture and extracting DNA from the cell pellet using MagMAX Microbiome Ultra Nucleic Acid Isolation Kit for KingFisher Flex (Thermo Fisher). Genomic DNA was then sent to Genewiz (San Diego, CA) for Sanger sequencing of the ITS using primers TW81 and AB28 (Goff et al. 1994). The ITS sequence for *Dunaliella* sp. 15-1 was submitted to the NCBI GenBank sequence database under accession number MN537907. Alternatively, heterotrophic strains were streaked onto 23%

MGM agar plates and submitted to Genewiz for Sanger Sequencing of the 16S rRNA gene using universal prokaryotic 16S primers 515F/806R (Walters et al. 2015). 16S sequences for the heterotrophic isolates will be submitted to GenBank prior to publication. ITS and 16S sequences were classified using the web-based RDP Classifier tool (Wang, Garrity, Tiedje, and Cole 2007), now only available as stand-alone application (https://github.com/rdpstaff/classifier), using a minimum bootstrap support value of 80%.

2.4 Culture conditions

From the identified strains, *Dunaliella sp.* 15-1 was selected as the representative *Dunaliella* strain for use throughout the experiment. An axenic culture of the strain was isolated in 2017 using the methods of Van Tol et al. (2017). This process included inoculation of a mixture containing 10 mL of L1-121 ppt media and 100 µL of a 100x antibiotic cocktail (Van Tol et al. 2017) (10 mL of Milli-Q, 50 mg streptomycin, 65 mg gentamycin, 20 mg ciprofloxacin, 2.2 mg chloramphenicol, 100 mg ampicillin) with 200 µL of a turbid *Dunaliella sp.* 15-1 culture. After 2 weeks the culture was refreshed with antibiotics. The strain was confirmed as axenic by plating 100 µL onto a 10% Difco 2216 Marine Broth agar plate (BD Difco 212185, Becton Dickinson and Company, USA) amended with 100 ppt NaCl (2216-100) and incubated in the dark for 4 weeks (Le Chevantona et al. 2013).

The axenic *Dunaliella sp.* 15-1 used in this experiment was refreshed from these existing axenic *Dunaliella* cultures using the following method: inoculation of 20 mL of L1-93 ppt media with 200 µL of existing axenic *Dunaliella* culture. After 2 weeks of growth, the culture was examined for turbidity and color. Due to the difficulty growing the cultures, multiple refreshes were performed. When little growth was observed, changes to the methodology were introduced including new nutrient stocks, increased sterilization measures, and a more concentrated culture.

These changes proved fruitful and new cultures began to appear more turbid until a significantly green, opaque culture was created. An aliquot of the turbid culture was then treated with 200 µL of a 100x antibiotic cocktail (Figure 2A) and then refreshed in fresh media two weeks later (Figure 2B). To ensure that the newly grown *Dunaliella* culture was truly axenic, without any bacterial or archaeal strains, 100 µL were streaked on 10% Marine Difco agar plates with 100 ppt NaCl (Figure 2C) and incubated over the course of the co-culture experiment. Simultaneously, a PCR was performed (Figure 2C) with primers 799F and 1392R to look for bacteria using the 16S gene without amplification of the chloroplast and primers 340F and 1000R to amplify the 16S gene and identify any potential archaea present in the sample (Hanshew, Mason, Raffa, and Currie 2013; Gantner, Andersson, Alonso-Saez, and Bertilsson 2011).

Heterotrophic strains used in this study (Table 2) were selected based on their rapid growth rate, ease of cultivation, and availability of Whole Genome Shotgun (WGS) sequence data (unpublished). All 8 isolate cultures were derived from glycerol stocks created in 2019. Using a 1:100 dilution, 50 µL of bacteria/archaea stock were used to inoculate 5 mL of 23% MGM (Figure 2D). The following cultures were then stored in a 37^{o}C incubator with no overhead light for 2 weeks. To check for contamination, 100 µL of each isolate was evenly distributed onto a 23% MGM agar plate (Figure 2E). Plates were subsequently incubated in a 37^{o}C incubator with no overhead light until substantial growth was observed and morphological inconsistencies could be assessed.

2.5 Polymerase chain reaction conditions for verifying axenic growth

A 5 mL sample of the *Dunaliella* culture, used in the co-culture experiment, was centrifuged at 5000 rpm for 10 min to pellet the algae (Jagielski et al. 2017). The supernatant was then removed, and the pellet was pipetted into a KingFisher bead-beating tube. Samples were

stored at -80°C. DNA was extracted from the pellet using the MagMax Micro-biome Ultra Nucleic Acid Isolation Kit (ThermoFisher) and KingFisher Flex (ThermoFisher). DNA concentrations were measured using Qubit dsDNA high-sensitivity assay. Extracted DNA was stored at -80°C. PCR was carried out on the extracted genomic DNA using the following conditions. Each PCR reaction contained 13 µL of PCR nuclease free water, 10 µL of Hot master mix (iTAQ DNA Polymerase, dNTP, MgCl2, Buffer)(ThermoFisher), 0.5 µL of each primer, and 1 µL of DNA in a final volume of 25 µL. Two PCRs were performed with one looking at potential bacterial contamination used 16S universal primers with modifications to remove potential chloroplast amplification of the *Dunaliella* (799F: CMGGATTAGATACCCKGG,1392R: ACGGGCGGTGTGTRC) (ThermoFisher) and the other investigating archaea contamination by using the 16S universal primers for archaea (340F: CCCTAYGGGGYGCASCAG, 1000R: GGCCATGCACYWCYTCTC) (ThermoFisher) (Hanshaw, Mason, Raffa, and Currie 2013). Each PCR was performed with a positive control (ZymoBIOMICS Microbial Community Standard, archaea 025 DNA) in triplicate, the sample DNA in triplicate, and a negative control in duplicate with -1 µL of nuclease free water in replacement of DNA. PCR was carried out in a thermocycler with the following conditions 95 °C for 3 min, 30 cycles of 98 °C for 20 s, 50 °C for 15 s, 72 °C for 15 s, and a final elongation of 72 °C for 1 min holding at 4°C. Following completion of the last cycle, 10 µl of reaction mix + 2 µL of 6x orange loading dye SYBR green (ThermoFisher) were analyzed on an agarose gel (1% w/v 1XTris-acetate –EDTA buffer with 1g of agarose) and visualized using a UV lamp.

Table 2. Isolate collection and enrichment

Date Collected	Isolate	Classification	Enrichment Media	Site	Brine/Sediment
2019.08.07	023	*Halolamina sp.*	23%MGM	6	Brine
2019.08.07	024	*Halorubrum sp.*	23%MGM	6	Brine
2019.08.07	025	*Halolamina sp.*	23%MGM	6	Sediment
2019.08.07	027	*Halorubrum sp.*	23%MGM	6	Sediment
2019.08.07	030	*Halomonas sp.*	23%MGM	8	Sediment
2019.08.07	031	*Halolamina sp.*	23%MGM	8	Sediment
2019.08.07	032	*Halorubrum sp.*	23%MGM	8	Sediment
2019.08.07	034	*Halorubrum sp.*	23%MGM	8	Sediment
2017.10.06	15-1	*Dunaliella sp.*	L1-121ppt	0	Brine

2.6 Optimization of co-culture conditions

To determine an effective salinity range for the co-culture experiment, *Dunaliella* was grown under six equidistant salinity conditions (40 ppt – 196 ppt) in triplicate. Using a 24 well black-walled microplate with lid (Greiner), 18 mL of L1 media, amended to the desired salinity for each treatment, was inoculated with 2 mL of *Dunaliella* and incubated at 22^{o}C with a 12-hour light/12-hour dark cycle. For the next 2 weeks, chlorophyll *a* was measured daily using a Bio Tek Synergy H1 Plate Reader at excitation wavelength 405 nm and emission wavelength 686 nm.

An optimal incubation temperature of 22^{o}C or 30^{o}C was then investigated using the following experiment: A 1:10 dilution culture (2 mL of *Dunaliella* in 20 mL of L1-93 ppt) was incubated at either 22^{o}C or 30^{o}C with a 12-hour light/12-hour dark cycle. Every day for 2 weeks a sample of 200 µL from this culture was distributed into a well of a sterile, black walled, 96 well plate 3 times and chlorophyll *a* measurements were read on a microplate reader

2.7 Dunaliella growth in co-culture with increasing salinity

Seven black-walled 24 well microplates were prepared with 162 incubations, consisting of 9 treatments (8 heterotrophic isolates plus 1 axenic control) × 6 salinity conditions × 3 replicates (Figure 2F, Table 3). Each well containing 2 mL of the appropriate media was inoculated with 20 µL of *Dunaliella* and 20 µL of a heterotrophic isolate. Row D of plate 7 was selected as the instrumental blank containing no *Dunaliella* or heterotroph and rows A-C of plate 7 were selected as the control containing only *Dunaliella* and media. Plates were topped with a lid and wrapped in electrical tape to minimize evaporation, then stored in a 30^{o}C incubator on a 12-hour light/12-hour dark cycle. For 2 weeks, two measurements were taken daily, approximately 6 hours apart, and were subsequently reduced to once a day between the hours of 12pm-5pm. All measurements ceased on the 30th day of the experiment.

The optical effects of hypersaline media on microplate reader fluorescence measurements were quantified by analyzing 3 different concentrations of *Dunaliella* (20 µL, 110 µL, and 200 µL in 2 mL of media) in 6 salinity treatments (40 ppt -196 ppt). A one-time chlorophyll *a* measurement was taken from 54 separate incubations (3 replicates x 3 concentrations x 6 salinities), and the mean and standard error were calculated using the 3 replicates of each treatment. Significance was then assessed with an ANCOVA (Analysis of Covariance).

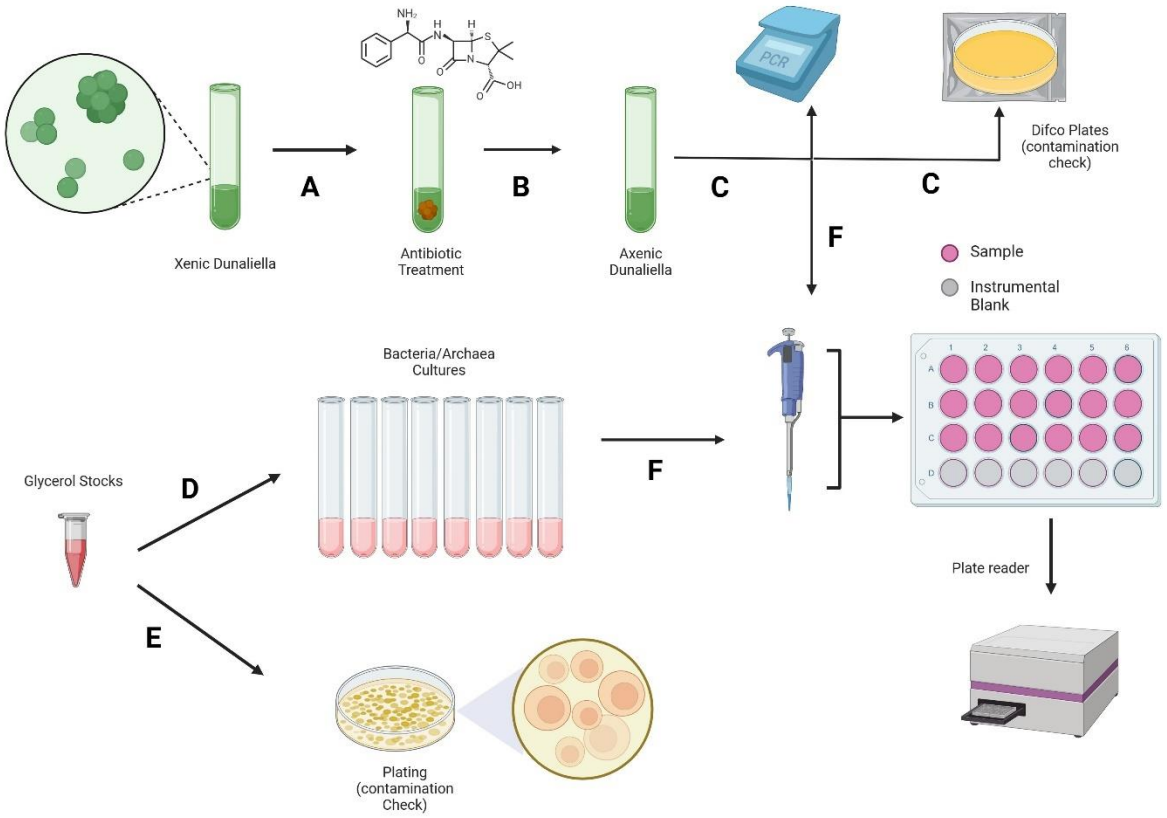

Figure 2. Representative workflow of co-culture experiment. **A:** A healthy Xenic *Dunaliella* culture is refreshed, treated with antibiotics, and left to grow for 2 weeks into its exponential growth phase. **B:** The resulting axenic culture is refreshed in new antibiotic-free media. **C:** Axenic *Dunaliella* culture is plated on Marine Difco plates without light and additionally checked for contamination with PCR. **D:** Prokaryotic cultures for all 8 isolates are refreshed from their original glycerol stocks and incubated for 2 weeks. **E:** An aliquot from each glycerol stock is plated on 23% MGM and morphological abnormalities are assessed for potential contamination. **F:** Resulting *Dunaliella* and prokaryotic cultures are pipetted onto a 24 well plate in triplicate with one row of instrumental blanks. Chlorophyll measurements are then read daily by a plate reader.

Table 3. Ion concentrations of culture media.

Media Type	Mg+ (ppt)	K+ (ppt)	Cl- (ppt)	Ca2+ (ppt)	Na+ (ppt)	TDS(ppt)
L1-40ppt	1.33	0.43	22.88	0.44	13.01	39.63
L1-76pt	1.28	0.41	44.72	0.42	27.24	75.56
L1-93ppt	1.26	0.40	55.04	0.41	33.96	92.54
L1-109ppt	1.23	0.40	64.99	0.41	40.45	108.9
L1-121ppt	1.22	0.39	72.21	0.40	45.16	120.8
L1-140ppt	1.19	0.38	83.84	0.39	52.73	139.9
L1-169ppt	1.15	0.37	101.4	0.38	64.19	168.9
L1-196ppt	1.11	0.36	117.9	0.37	74.91	195.9
Seawater	1.34	0.43	20.03	0.44	1.34	34.94
23% MGM	4.69	2.45	106.5	0.13	4.69	185.8

Total dissolved solids (TDS) were calculated by summing all ion concentrations. Ion concentrations were derived from the major ion composition of 35 ppt Standard Seawater concentrations taken from Millero, Feistel, Wright, and McDougall (2008). Salinity concentrations for L1 media were determined by the following equation (S + 35)/ (S + W)) *1000. S is the added salt in grams and W is the density of 35 ppt seawater. W was reported as 1024g/L in all calculations.

2.8 Dunaliella growth in co-culture with isolates 025 and 027 for metabolomic analyses

Co-cultures were initially created in triplicate with 98 mL of L1-140 ppt media and 1 mL each of *Dunaliella* (originally grown in 30^{o}C and refreshed) and 1 mL of one heterotroph (025, 027). In a similar 100 mL glass bottle, 98 mL of L1-140 ppt media, 1 mL of *Dunaliella*, and 1 mL of 23% MGM acted as a control. Due to likely a media or glassware problem, the cultures showed no growth after two weeks, and the experiment was redone. For the next iteration, the glass bottles were exchanged for acid washed 250 mL culture Erlenmeyer flasks with filter caps and new media

was made. The cultures were then set to grow under similar conditions as the first iteration in an incubator set at 30°C with a 12-hour light/dark cycle for 2-4 weeks (Figure 3).

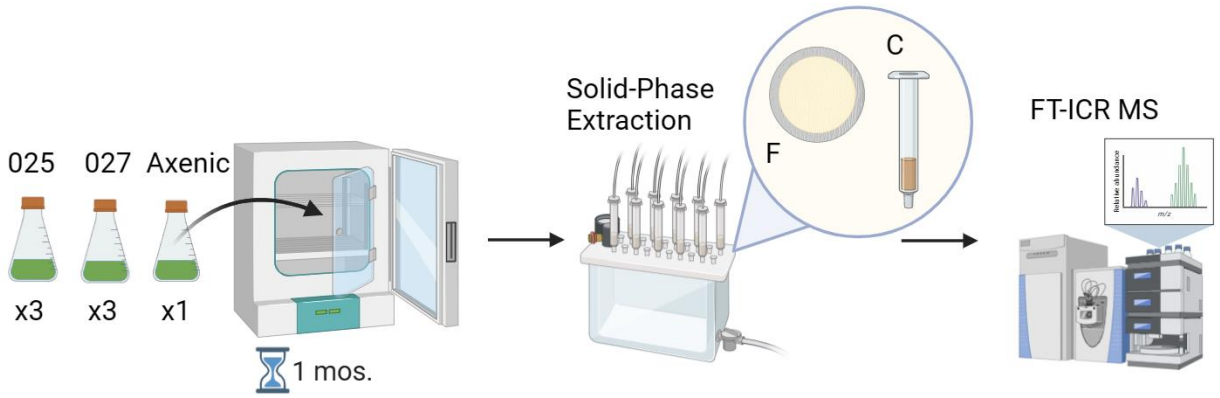

Figure 3. Metabolomic workflow of 025 and 027 co-cultures. Seven samples were divided into three treatments (3 samples for co-culture 025, 3 samples for co-culture 027, and 1 sample for Axenic). All samples were then incubated for 1 month and then processed through solid phase extraction for the collection and isolation of metabolites. Two samples (C= Column, F=Filter) were collected for each replicate of each treatment and sent off for FT-ICR mass spectrometry.

2.9 Isolation and collection of metabolites

Cultures were processed in their stationary growth phase by solid phase extraction (SPE). Prior to the SPE, filter holders were acid washed and Milli-Q ultrapure water (Millipore) was run through the 100 mL syringes and the filter holders. Filter holders were then loaded with combusted 47 mm GFF filters (Whatman) and attached via Teflon tubing to a 3 cc, 100 mg, labeled Agilent bond elut ppl column. Simultaneously, another 250 mL Erlenmeyer culture flask was filled with 100 mL of Milli-Q and left to warm up to the incubated 30°C temperature. Once ready, the incubated Milli-Q water was processed via SPE and the filter and column were collected as instrument blanks. The filter was folded with biomass on the inside and packaged into an aluminum envelope. The column was placed into a sterile plastic bag, and both were momentarily stored at -20°C. All other cultures were acidified to PH 2 using 50% HCl (~100 µL), and the columns were

activated by being flushed with 2 mL of MeOH. Acidified samples were added to the syringes/reservoirs and were flushed through the SPE manifold. Once the sample was fully flushed, 5 mL of 0.01M HCl was added to each reservoir and flushed through. Filters and columns were then collected similarly to that of the blank sample and were all moved to be stored at -80^{o}C. A few days later, the samples were shipped off to the Tfaily lab (University of Arizona, Tucson, AZ) for processing with FTICR-MS.

2.10 FT-ICR mass spectrometry

A Bruker 9.4-Tesla FTICR-MS was used for high resolution characterization of organic matter (OM). After SPE, eluants in methanol were introduced directly to a standard Bruker electrospray ionization (ESI) source operating in negative mode. Instrument parameters can be found here (AminiTabrizi, Dontsova, GrafGrachet, and Tfaily 2022).

Prior to analysis, the raw spectral peak intensity data was adjusted to account for potential contaminants and artifacts by removing signals found in the procedural blanks from the other samples. Compounds found in the column blank were removed from other column samples and vice versa for filter samples. The adjusted peak intensity data was then log-transformed and processed through the metabodirect pipeline (https://github.com/Coayala/MetaboDirect) using a minmax normalization method, and Treatment (025, 027, D) and Type (C, F) grouping variables. A minmax normalization method was selected based on the additional test_normalization script available which uses a modified version of the spans_procedure function in the R package, PMART. The metabodirect pipeline then filtered out peaks based on isotopic presence (13C peaks) and error in ionized mass versus neutral mass (0.5ppm). After filtering, 12016 peaks remained out of 122043.

2.11 Statistical analysis for growth kinetics

Chlorophyll *a* measurements for the salinity experiment were used to estimate *Dunaliella* growth curve parameters using the R package, growthrates. With the fit_easylinear function, five consecutive data points were selected to determine the maximum growth rate from the log-linear part of the growth curve. Five data points were used to determine growth rate as it resulted in the least difference between replicate growth rates of each treatment. A one-way ANOVA was then conducted to investigate the effect of salinity on growth rate followed by a Tukey Post Hoc analysis to examine differences in that effect between treatments.

For temperature, chlorophyll *a* measurements were used to estimate *Dunaliella* growth curve parameters using the R package, growthcurver. With the function SummarizeGrowth, a logistic regression was fitted to each treatment replicate, and the calculated growth rates were recorded in a new dataset. Based on the fit, outliers including the first point of every replicate and the second to last point for 22:2 (Treatment:Replicate) were excluded. A One-way ANOVA was then run on the new dataset examining the effect of temperature on growth rate followed by a Tukey Post Hoc test.

Growth rates of Dunaliella for each of the treatment conditions in the initial co-culture were derived through the R package, growthrates. Using the function fit_easylinear, fourteen consecutive data points from the logarithmically transformed data were employed to conduct a linear regression which assessed the slope of the growth phase. The quality of easylinear's growth rates were then validated through additional manual calculations. Such calculations involved performing a linear regression on a time span, specified by the researcher, that individually fit the assumed growth phase for each replicate of each treatment. Means of each treatments' replicates along with their standard errors were calculated and compared to those calculated using easylinear.

The effect of co-culture treatment on growth rates across the various salinities was modeled through the use of two natural cubic spline functions (df=2), one taking into account treatment and salinity (alternate hypothesis), the other only including salinity (null hypothesis). A one-way ANOVA was then used to compare the two models for significance of treatment.

2.12 Statistical analysis for metabolomics analysis

Normalized peak intensities of the metabolomics co-culture data were transformed into Manhattan distances using the vegdist function in the R package, vegan to perform permutational analysis of variance (PERMANOVA) and non-metric scaling of normalized intensities (NMDS) (Anderson 2017; Kruskal 1964). Further exploratory pairwise comparisons within grouping variables were conducted using the custom plot_van_krevelen and plot_comp_bar R functions from Metabodirect and the R package, ggven. Resulting analyses included visualization of metabolite class compound groupings through bar and van krevelen plots and a Venn diagram displaying shared versus unique metabolites between groups. Significance in metabolite abundance was confirmed first by calculating the z-score of the axenic control relative to treatment groups for each peak, using the formula $z_{control} = (X_{control} - \bar{X}_{all}) / SD_{all}$. P-values to determine significance were then calculated by bootstrapping all peaks 10,000 times, creating a z-score distribution for each peak, and then assessing the probability of obtaining a z-score as extreme or more extreme than the true control z-score from the distribution of z-scores observed in the bootstrapped samples. A significant difference was assigned to any peak that had a p-value of less than 0.05. Bootstrapping was used to most accurately assess significance given the singular replicate for the axenic control. Significant peaks were then annotated through queries of the KEGG database (Kanehisa and Goto 2000) using the R package, KEGGREST, and through matching of monoisotopic mass, molecular formula, and class using the Pubchem (Kim et al.

2023), Chemspider (Pence and Williams 2010), NIST (National Institute of Standards and Technology 2001) and RCSB PDB (Berman et al. 2000) databases. Further visualization of the data was generated through Molecular transformation networks for each sample in Cytoscape (Shannon et al. 2003) and colored based on their molecular class. Nodes represent peaks detected and edges represent putative chemical transformations between the nodes (Fudyma et al. 2019; Breitling et al. 2006; Longnecker and Kujawinski 2016).

RESULTS

3.1 Dunaliella growth rates with increasing salinity

Prior to the co-culture experiment, salinity and temperature parameters were selected based on the results of two preliminary experiments depicted in Figure 4. To ensure the optimal salinity of *Dunaliella sp.* 15-1 was sufficiently encompassed in the selected range (40 ppt - 196 ppt), growth rates were calculated from the chlorophyll values collected daily over the course of two weeks for each salinity condition. As illustrated by Figure 4A, mean growth rates of *Dunaliella sp.* 15-1 were found to be statistically different (ANOVA; $p < 0.0001$) between 40 ppt and salinities above 109 ppt. While the growth rates between 140 ppt and 109, 169, and 195 ppt were not statistically different, a parabolic trend persisted, with the growth rate peaking at 140 ppt and then slowly descending at higher salinities. An experiment investigating *Dunaliella sp.* 15-1 growth at 30°C and 22°C found a significantly higher growth rate at 30°C (ANOVA; $p = 0.0304$) (Figure 4B). This was important to the outcome of the co-culture since a higher temperature could now be used that didn't impede *Dunaliella* growth and benefited prokaryotic growth.

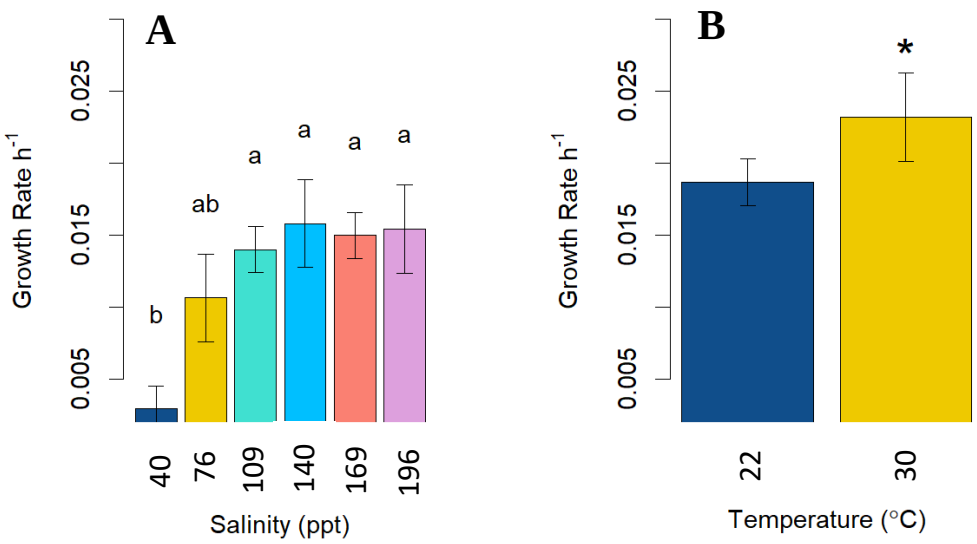

Figure 4. Co-culture parameter analysis of temperature and salinity. **A:** Bar plot comparing mean *Dunaliella sp.* 15-1 growth rates at different salinities; n=3. Differences in letters indicate statistical significance between treatments ($p < 0.05$). **B:** Bar Plot comparing mean growth rate of *Dunaliella*15-1 at two different temperatures; n=3. Statistical significance is designated with an asterisk ($p < 0.05$).

Growth rates for the eight co-cultures and axenic culture (Figure 5) demonstrated higher growth rates in the majority of co-cultures (6/8) compared to the axenic in salinities above 140 ppt. Co-cultures 032 and 030, however, exhibited decreased growth over time at 140 ppt but exceeded growth rates in the axenic culture at salinities above 169 ppt. Similar findings were observed at salinities of 108 ppt and lower, with only two co-cultures (031 and 032) having growth rates lower than that of the axenic culture.

To ensure contamination was not a factor regarding the *Dunaliella*/prokaryote strains, 100 µL of each isolate was spread onto either a 23% MGM agar plate (for prokaryotes) or a 2216 Marine Difco agar plate (for axenic *Dunaliella).* The resulting growth on the 23% MGM agar showed no morphologically distinct colonies, and no growth was observed on the Marine Difco plate in the absence of light, confirming the purity of the cultures. Optical effects of salt

concentration on fluorescence measurements were assessed to examine the potential effects of evaporation on reported chlorophyll *a* measurements. Fluorescence measurements for three algal concentrations in six different salinities were analyzed. Results showed a significant effect of salinity on fluorescence (ANCOVA; $p = 0.0002$), with no significant interaction between algal concentration and salinity ($p = 0.1716$). Therefore, evaporation likely had an effect on reported fluorescence measurements, but the effect size was small (0.25 Relative Fluorescence Units (RFU)/ ppt), and thus likely had minimal impact on downstream growth rates calculations.

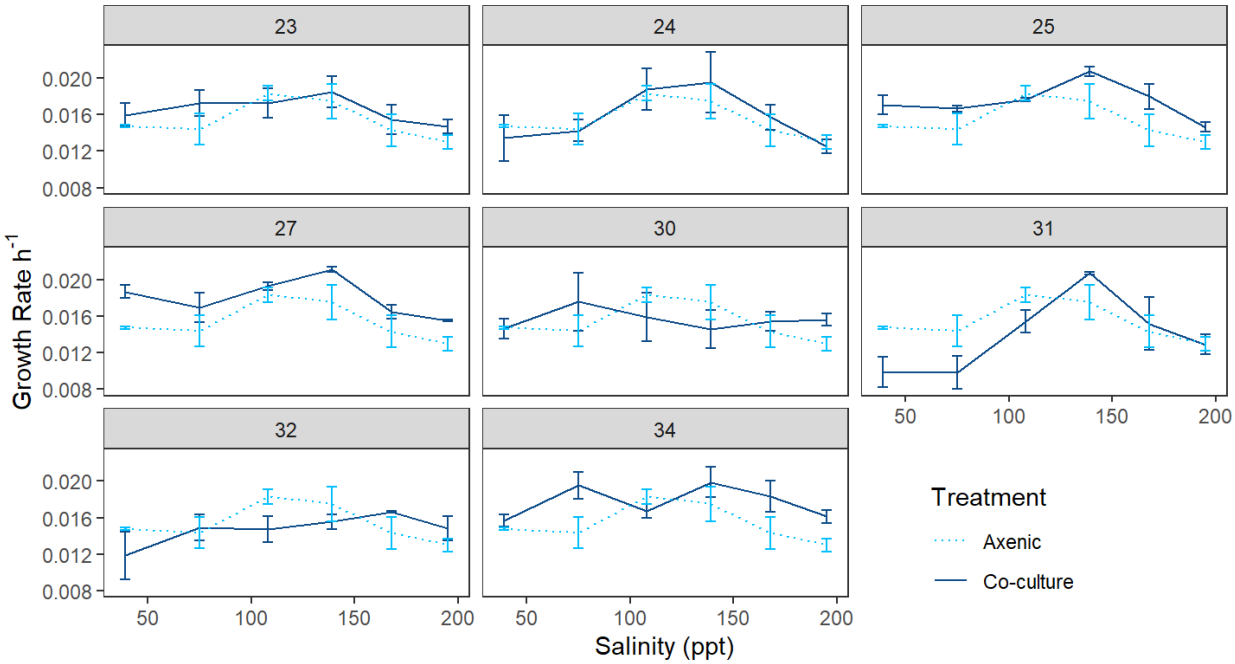

Figure 5. Growth rates of *Dunaliella* co-cultures vs axenic. Line graphs investigating mean growth rate (µg/Lh) of *Dunaliella sp.* 15-1 as a function of salinity (ppt) and facet wrapped by prokaryotic isolate. Co-cultures are represented by a solid line, and monocultures are represented by a dotted line.

3.2 Modeling effects of treatment

To assess these findings for significance, the effects of salinity and treatment on *Dunaliella* growth rate were modeled using cubic spline functions. While multiple models were considered, a natural cubic spline model with 2 degrees of freedom was chosen based on a cubic relationship between salinity and growth rate and the continued presence of Runge's phenomenon in previous

models. Comparing the two models for the effect of treatment, 025 (ANOVA; p = 0.0469), 027 (ANOVA; p = 0.0126), and 034 (ANOVA; p = 0.0349) co-culture treatments resulted in a significantly improved model with a better fit to the data than the model only considering salinity. Due to the nature of the approach, significance regarding specific salinities could not be assessed. However, as shown in Figure 6, all three significant treatments performed better than the control in at least one or more salinities above 140 ppt. Co-culture 025 appeared to demonstrate a substantially improved growth rate for all salinities above 140 ppt with no crossover in standard error (SE) bars. Cultures 027 and 034 similarly showed substantial growth rates at higher salinities except for minor crossover in SE bars at one salinity concentration (140 ppt - 034, 169ppt - 027).

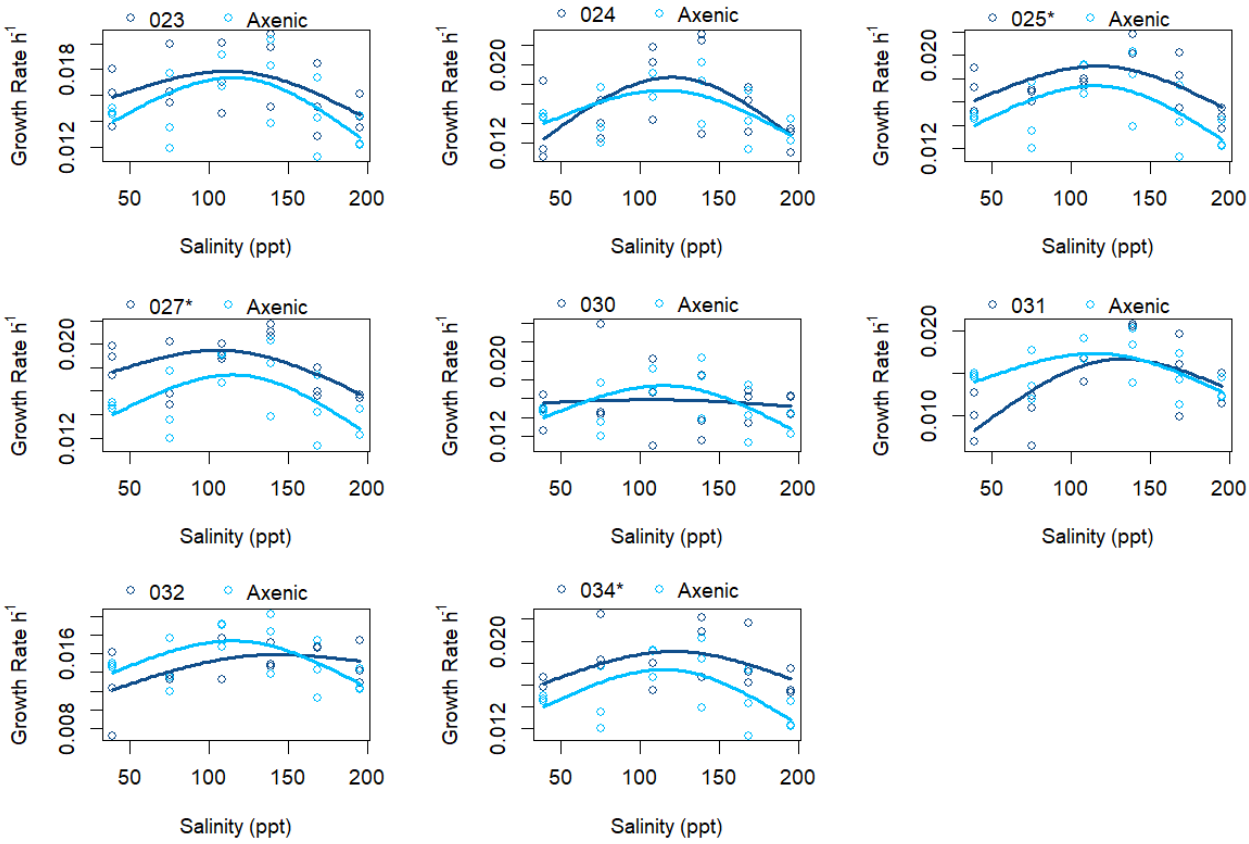

Figure 6. Cubic spline models comparing co-cultures and axenic. Each set of line graphs utilize natural cubic spline models (df =2) to assess growth rate as a function of salinity alone (dark blue) or as a function of both salinity and isolate treatments (light blue). Significance of isolate treatment compared to axenic is noted with an asterisk ($p < 0.05$).

3.3 Metabolomic profiles of 025 and 027 co-cultures

To investigate the mechanisms behind these improved growth rates, isolates 025 and 027 were selected for metabolomic analyses. Comparing the co-cultures against the axenic metabolites, Figures 7A and 8A show an increased presence of all classes of metabolites in the two co-culture treatments compared to the axenic control. These increases are most significant for lipids, proteins, lignins, and unsaturated hydrocarbons. It is interesting to note that between both co-culture treatments compared to the axenic, similar patterns in distribution of metabolites by class begin to emerge. In Figures 7C and 8C looking at molecular class percentages for co-culture, axenic all displayed notable percentages of ~80% split between those 4 classes and limited differences.

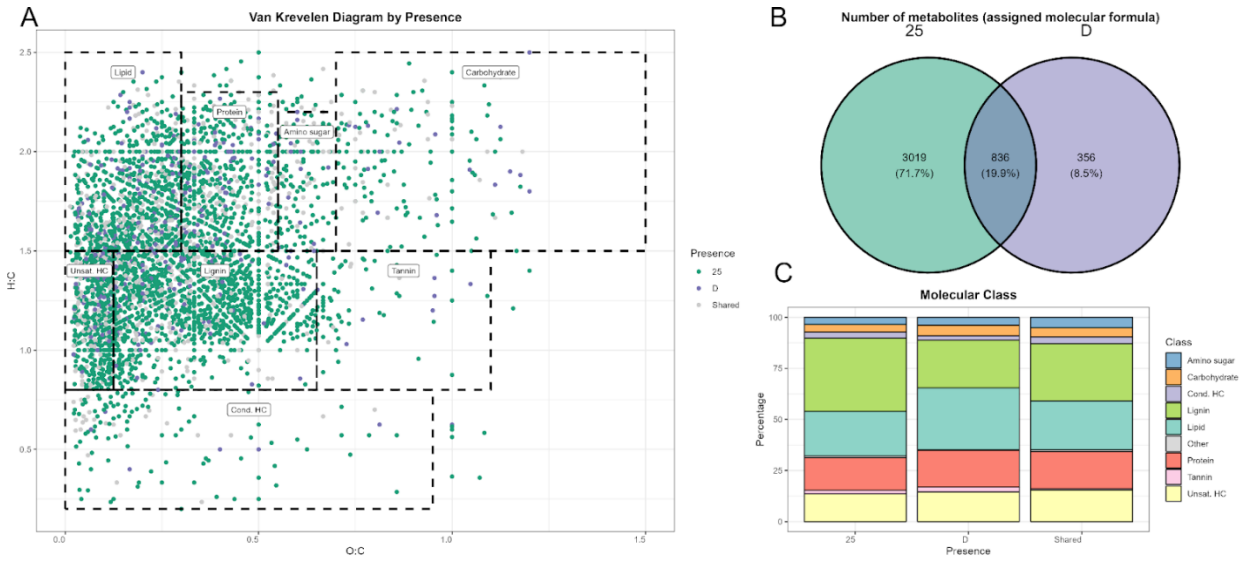

Figure 7. Metabolomic profile of 025 co-culture vs axenic. **A:** Van Krevelen diagram with color coded metabolites based on presence in the 025 co-culture (green), in the Axenic (purple), and in both cultures (grey). Black dashed lines set class boundaries based on the ratio of hydrogen to carbon (H:C) and oxygen to carbon (O:C). **B:** Venn Diagram showing the number of shared and unique metabolites within the two cultures. **C:** Bar Plot illustrating the percentage of each class within each culture and between both cultures.

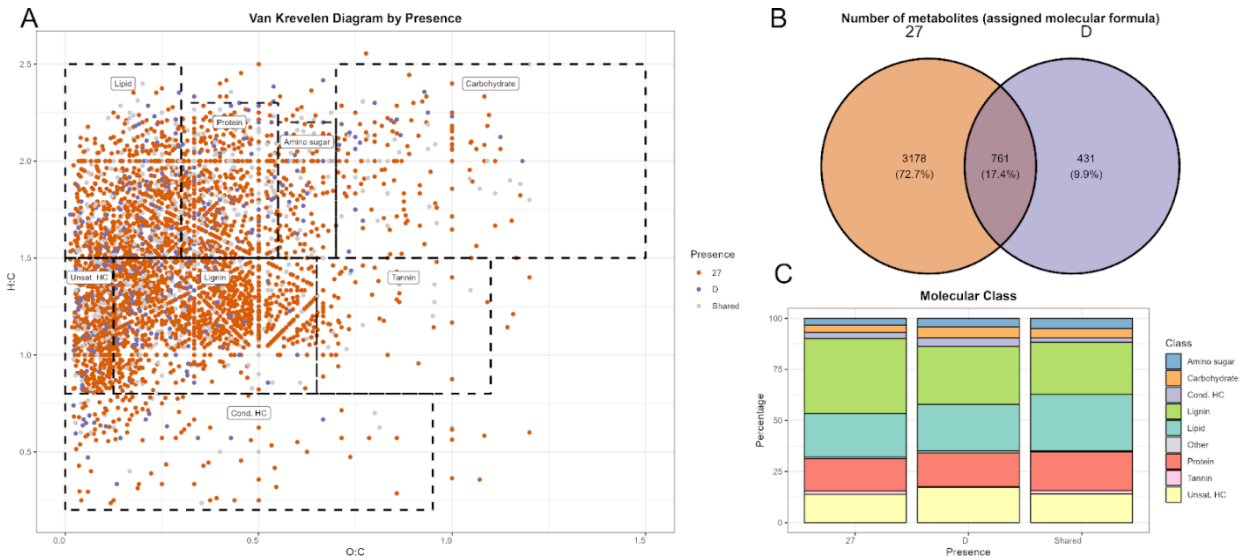

Figure 8. Metabolomic profile of 027 co-culture vs axenic. **A:** Van Krevelen diagram with color coded metabolites based on presence in the 027 co-culture (orange), in the Axenic (purple), and in both cultures (grey). Black dashed lines set class boundaries based on the ratio of hydrogen to carbon (H:C) and oxygen to carbon (O:C). **B:** Venn Diagram showing the number of shared and unique metabolites within the two cultures. **C:** Bar Plot illustrating the percentage of each class within each culture and between both cultures.

3.4 Identification of significant metabolites

Further metabolomic analysis of *Dunaliella sp.* 15-1 co-cultures revealed significant differences in metabolite expression compared to the axenic culture. Out of 4029 metabolites detected and analyzed, 87 were identified as significantly different between the co-culture treatments and the axenic control ($p < 0.05$), with 84 exhibiting decreased expression and 3 exhibiting increased expression in the co-culture treatments (Figure 9, Figure 10). Relative abundances of all 87 compounds are shown in Figure 10 with 33 found in both *Halolamina* (025) and *Halorubrum* (027) treatments, 26 found only in *Halolamina* treatments, and 27 found in *Halorubrum* treatments. Significant compounds also consisted of 4 aminosugars, 4 carbohydrates, 4 condensed hydrocarbons, 25 lignins, 24 lipids, 2 others, 16 proteins, and 7 unsaturated fatty acids. Beyond class distinction, only nine of the downregulated (84) compounds and all of the

upregulated (3) were able to be matched with a known compound. These annotated compounds, identified through matching of monoisotopic mass and molecular formula, are recorded in Table 4 with their associated classes. In addition to these compounds, 4 compounds with a nearly significant p-value were also investigated for their increased expression in the treatment conditions (Figure 9, Figure 10) and recorded in Table 4.

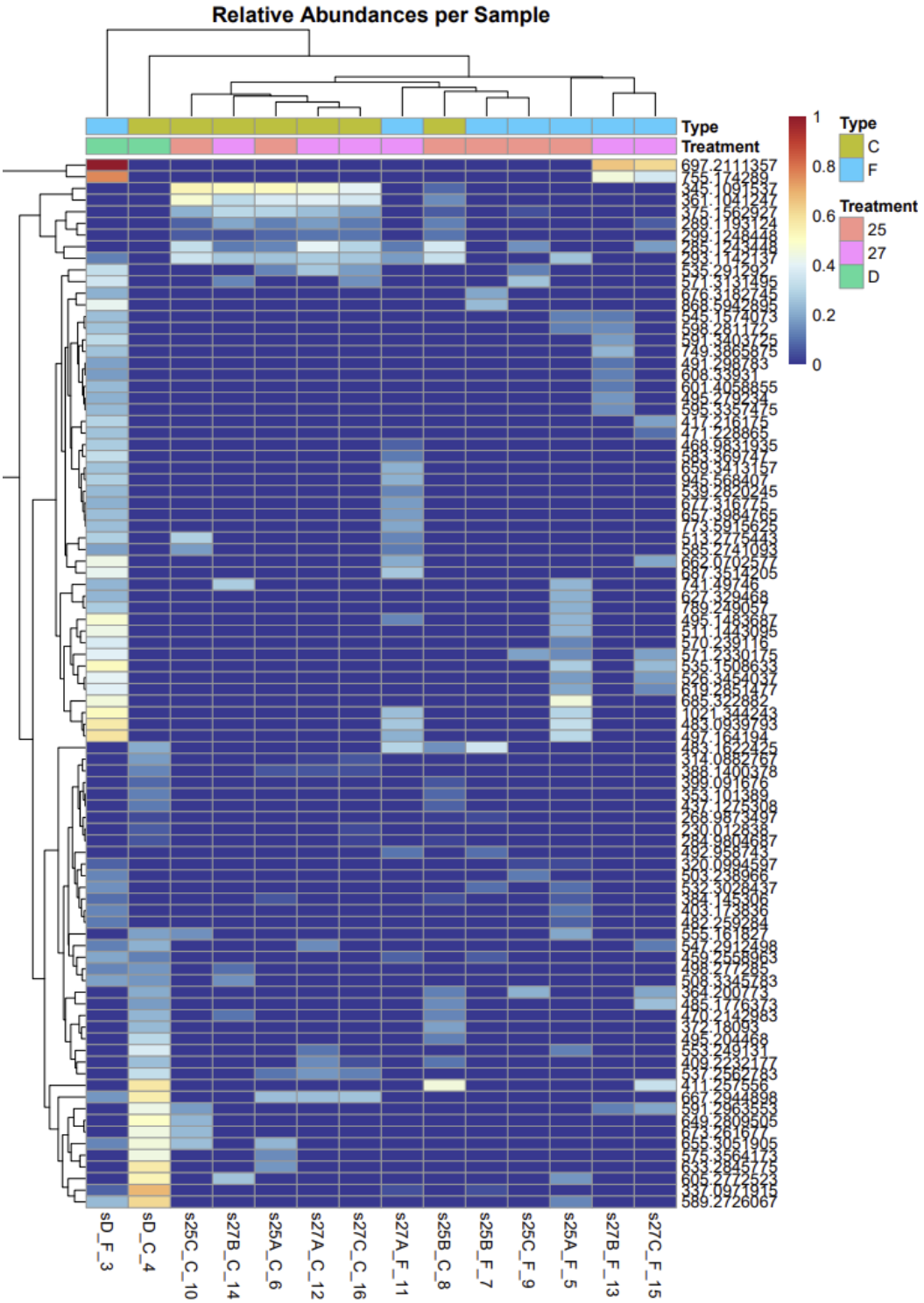

Figure 9. Heatmap analysis of significant metabolites. Higher relative abundance is shown in red and lower abundance is shown in blue. The molecular weight of all significant metabolites is listed along the y-axis and samples are listed along the x-axis. Type and Treatment of all samples are specified under the x-axis dendrogram.

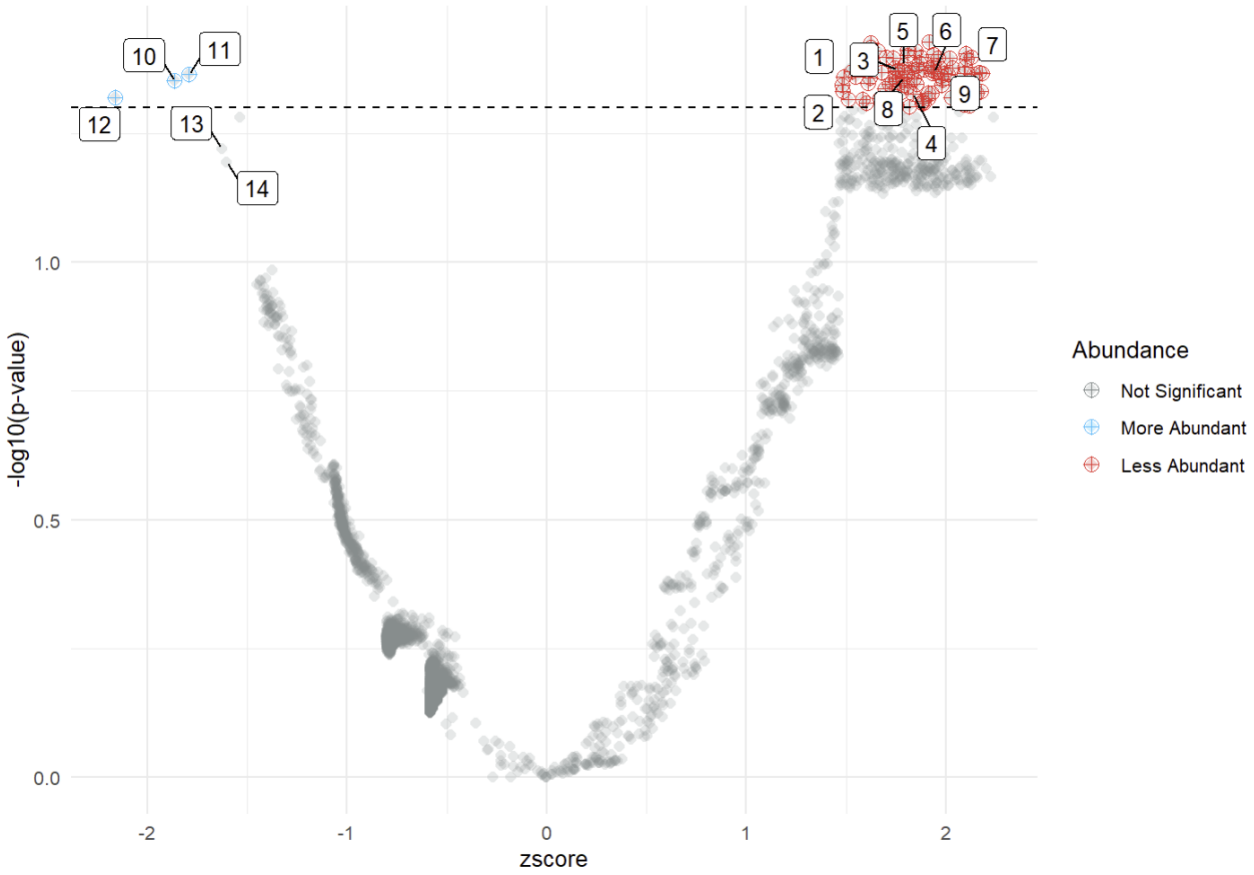

Figure 10. Volcano plot of differentially abundant metabolites. Significant differences in metabolite expression relative to the axenic control are marked in red (overexpressed in axenic, *i.e.* less abundant in co-culture treatments) or blue (underexpressed in axenic, *i.e.* more abundance in co-culture treatments). All other non-significant metabolites are indicated by the color grey. The x-axis is the z score or the standard deviation of the metabolite peak in the control sample from the mean of the metabolite peaks in all samples, and the y-axis is the negative log 10 of the p-value. The grey dashed line highlights the threshold of significance ($p<0.05$).

Table 4. Annotated metabolite compounds. All compounds correspond to Figure 10.

#	Metabolite	Class	P-Value
1	Ethanolamine Phosphate	Lipid	0.0436
2	5-pentanoic acid	Unsaturated HC	0.0466
3	2-methylhexanoic acid	Lipid	0.0424
4	N-Cysteine	Protein	0.0471
5	Cymarin	Protein	0.0414
6	Bis(L+)-tartaric acid	Protein	0.0427
7	Rhodexin A	Protein	0.0393
8	Forskolin	Protein	0.0438
9	β-D-glucopyranuronic acid	Lignin	0.0435
10	2-methyl-3-propanoate	Protein	0.0444
11	Succinic Acid	Lignin	0.0432
12	Sarcosine	Lignin	0.0478
13	L-Glycine	Protein	0.0601
14	L-Proline	Protein	0.0640

HC = Hydrocarbon

DISCUSSION

From the initial co-culture experiment, the improved growth rate of *Dunaliella* in co-culture compared to the axenic culture demonstrated a clear positive effect of three heterotrophic prokaryotes on the microalgae's growth. Further analyses accounting for this treatment effect when examining the relationship between salinity and growth rate indicate that the positive effects brought about by the prokaryotes' presence were not due to chance alone. Therefore, a symbiotic relationship likely exists between *Dunaliella* and the three isolates (025, 027, and 034). This relationship is either mutualistic, where both organisms benefit, or commensalistic, where one benefits while the other is unaffected.

In analyzing the metabolomic profiles of the larger co-culture experiments, potential mechanisms by which the prokaryotes benefit the algae are postulated. While only a total of fourteen metabolites were successfully identified at a Level 2 or higher, according to the Metabolomics Standards Initiative (Sumner et al. 2007), key similarities in affected biological pathways were observed.

One of the main affected pathways is sodium and potassium ion transport. In high sodium environments, microalgae such as *Dunaliella maritima* utilize an ATP dependent sodium efflux method through Na+/K+ ATPase proteins in order to maintain K+/Na+ homeostasis by limiting the accumulation of sodium ions and the depletion of potassium ions (Hussian et al. 2021; Li et al. 2023; Kumari and Rathore 2019). In such conditions, the upregulation of ATP production is important for efficient functioning of Na+/K+ ATPase, as it ensures sufficient energy is supplied to drive the active transport of both ions. Upregulation of ATP is linked to an upregulation of compounds involved in the TriCarboxylic Acid (TCA) cycle (Keil et al. 2023).

As succinic acid is a key intermediate in the TCA cycle, an increase in succinic acid signifies heightened TCA cycle activity resulting in increased ATP production (Liu et al. 2022). This increase in ATP availability enhances the efficiency of Na+/K+ ATPase and ultimately results in better ion transport and osmoregulation for the cell. Furthermore, this mechanism is supported by the inhibition of cymarin, a cardiotonic steroid primarily used to inhibit Na+/K+ ATPase activity by binding extracellularly to alpha subunit of the antiporter (Lowndes, Hokin-Neaverson, and Ruoho 1984). Therefore, it can be concluded that the presence of *Halolamina* (025) and *Halorubrum* (027) species in co-culture with *Dunaliella* may be improving *Dunaliella's* growth through enhanced ion transport and osmoregulation.

Further support for increased ion transport efficiency as a mechanism for improved growth is provided by the enhanced presence of propanoate in treatment conditions. Increases in propanoate have been linked to an upregulation in TCA molecules such as citrate synthetase and succinic acid in addition to glycerol-3-phosphate (Wang et al. 2016). Therefore, upregulated propanoate metabolism has been associated with amplified levels of ion transport in conjunction with improved osmoregulation through glycerol synthesis and enhanced succinate content, the anionic form of succinic acid (Wang et al. 2016). This effect has been especially observed in studies concerning the tolerance of *Dunaliella* to hypersaline conditions (Wang et al. 2016).

Similar to the increases in succinate content and inferred increases in glycerol synthesis from upregulated propanoate metabolism, *Dunaliella* is known to accumulate additional compatible solutes like amino acids to help with osmoregulation in hypersaline environments (Lv, Qiao, Zhong, and Jia 2017). These amino acid solutes, such as sarcosine and glycine, can act as osmoregulators by accumulating in the cytoplasm and moving across the semipermeable membrane as needed to balance the intracellular and extracellular environments (Lv, Qiao, Zhong,

and Jia 2017). Due to their low molecular weight, solubility, and zwitterionic chemical properties, these compounds result in minimal enzyme inhibition and can even stabilize surrounding proteins (Lv, Qiao, Zhong, and Jia 2017). This stabilizing effect is particularly important as *Dunaliella* undergoes drastic shifts in protein abundance under high salinity conditions, with studies like Keil et al. (2023) identifying 691 proteins significantly upregulated after *D. tertiolecta* was exposed to hyper salinity for 24 hours. Interestingly, the amino acids sarcosine and glycine are precursors for the synthesis of another osmoprotectant known to be accumulated under severe salt stress, glycine betaine, which is formed through a three-step methylation process using S-adenosylmethionine as a methyl donor (Weinisch et al. 2018; Mishra, Mandoli, and Jha 2008).

Building on the role of amino acids like sarcosine and glycine as compatible solutes for osmoregulation, proline is another important compatible solute utilized by *Dunaliella* as an osmoprotectant and metabolite in abiotic stress response. Under conditions of sulfur deprivation and phosphorous depletion, *Dunaliella* exhibited upregulated levels of proline (Lv, Qiao, Zhong, and Jia 2017). Moreover, following long term exposure to hyper salinity conditions, proline was found to be one of the most abundant amino acids accumulated within the cytoplasm of *Dunaliella* (Bombo et al. 2023). This may be partly due to proline's numerous attributes that aid stress response, including protecting cellular growth and photosynthetic efficiency by reducing free radicals (Ramachandran et al. 2023), stabilizing surrounding proteins similar to other compatible solutes, and influencing membrane permeability through a feedback mechanism involving fatty acid composition (Xiao, Zhang, Feng, Cui 2012). Notably, the abundance of unsaturated fatty acids was directly linked to the abundance of osmolytes such as proline, where higher proline levels resulted in an increase in unsaturated fatty acids (Young-Lee et al. 2014). This could explain why

only a small number of unsaturated hydrocarbons, like hexanoic acid, were found to be upregulated in the control compared to the treatments.

Based on the metabolites identified, it seems that lipid composition might be an important factor in the mechanism at work to improve *Dunaliella* growth in the presence of extreme salt stress. With lipid accumulation and shifts in composition shown to result in significant defenses against osmotic stress and a down regulation in the control of ethanolamine phosphate, hexanoic acid, Valeric acid, and a number of other lipids, and an increase in osmolytes, it can be inferred that lipid composition along with membrane fluidity may be significant in the improvement of *Dunaliella* growth in co-culture (Pandit, Fulekar, and Karuna 2017; El Halmouch 2019; Denich, Beaudette, Trevors 2003). Ethanolamine phosphate, one of the downregulated metabolites in the treatment condition, is involved in the synthesis of a phospholipid known as phosphatidylethanolamine (PE), a polar lipid in the lipid bilayer. Even though this is a PolyUnsaturated Fatty Acid, previous studies have shown that in salinities ranging from 1-3M NaCl, cells tend to suffer a significant loss in PE abundance due to disrupted cytoplasmic enzymes (Peeler et al. 1989). Similarly, as a short chain unsaturated fatty acid, Valeric acid can insert itself into the lipid bilayer and influence the fluidity of the membrane (Panda, Rangani, and Parida 2021). However, this compound too might be downregulated because of other systems at play. Valeric acid is known to have additional antimicrobial properties that might be inhibited by *Dunaliella* 's prokaryotic counterpart (Panda, Rangani, and Parida 2021).

A reduction in certain antimicrobial compounds to prevent any negative effects on the prokaryotic isolate might also explain the Down regulation observed in the treatments for both Rhodexin A and Bis L+ tartaric acid. As both compounds are involved in mechanisms that aim to disrupt bacterial cell membranes or otherwise slow growth and apoptose cells, it would be

reasonable to assume that those compounds would be inhibited in co-culture (Faig et al. 2015; Benjamin, Lucini, Jothiramshekar, and Parida 2019). These compounds could also be inhibited purely because they were unnecessary to the Dunaliella's cellular functioning. In the case of N-terminal cysteine, forskolin, and glucuronic acid, which all confer some sort of stress response, their production may have been an unnecessary expense of valuable resources by the *Dunaliella* and therefore inhibited in the treatment conditions (Zhang et al. 2004; Dai, Wang, Xu, Chen 2022; Shetty, Gitau, and Maróti 2019). It is worth noting that due to the lack of replicate samples of the control, it was easier to identify metabolites as significantly less abundant in the treatments relative to the axenic control. Therefore, while this might explain the inhibition of some compounds that are typically upregulated as a response to stress, this more so asserts the importance and value of the metabolites that were identified as more abundant in the treatments.

Spanning the fields of pharmacology, biofuels, cosmetics, wastewater, and astrobiology, *Dunaliella* is one of the most influential microalgae. In the wake of global warming and substantial deleterious developments in the cultivation of *Dunaliella* with increased salt stress, this thesis aims to analyze and better understand the ecological relationship between halophilic prokaryotes and *Dunaliella* and the use of that relationship in the improvement of future algal maintenance and growth. Utilizing the methods of co-culture experiments and metabolomic analyses, the results showed the formation of a symbiotic relationship between 3 of the 8 prokaryotes in sub optimal salinity treatments. To better understand this phenomenon, metabolites collected from co-cultures with these specific prokaryotes were assessed and identified. Findings of these analyses emphasized an increased upregulation of lipids, lignins, proteins, and unsaturated hydrocarbons with specifically 84 metabolites found to be significantly over expressed and 3 under expressed. Potential mechanisms explaining these results found that the majority of pathways improved by

prokaryotic presence were osmoregulation through increased concentrations of compatible solutes, increased efficiency related to ion transport, and increased membrane fluidity, These are all pathways representing vital cellular functions that are typically downregulated with extreme stress demonstrating the importance of co-cultivation of other halophilic organisms with *Dunaliella* for industrial purposes. It is recommended that future studies investigate larger samples of halophilic prokaryotes as not all form a positive association with *Dunaliella,* and that future efforts refine the metabolomic analyses used. For more accurate pathway analyses, it is recommended to perform additional transcriptomic analyses to relate gene expression to metabolite expression.

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