A NOVEL AGGREGATING GROWTH HABIT IN *DUNALIELLA* SPP. (CHLOROPHYTA, DUNALIELLALES)

By

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A NOVEL AGGREGATING GROWTH HABIT IN *DUNALIELLA* SPP. (CHLOROPHYTA, DUNALIELLALES)

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Abstract: Species of *Dunaliella* are known to aggregate in a palmelloid stage, but they can also aggregate in a previously uninvestigated manner. This perpetual aggregation occurs in isolates from substrates such as the benthos, supralittoral zone, gypsum crusts, or salt flats, a subset of *Dunaliella* which has not been sufficiently examined. Two such isolates, GSL-3A4 and GSL-3C2 from Great Salt Lake, Utah, were compared morphologically to the more common single cell habit of isolates GSL-12A4 and GSL-6/1. A method for assessing aggregation efficiency was developed. This work sets the foundation for a new series of discoveries regarding *Dunaliella* growth habit and desiccation tolerance after 110 years of research with the genus.

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CHAPTER I

INTRODUCTION

Dunaliella is a cosmopolitan genus of green alga found in bodies of water ranging from freshwater to euryhaline and even acidic bodies of water (Polle et al. 2009). Members of the genus have been proposed for the production of biofuels (Minowa et al. 1995). Certain species serve as a model system for studying halotolerance because of their production of glycerol as a compatible solute (Ben-Amotz & Avron 1973, Cowan et al. 1992, Pick 1998). Other species produce high levels of β-carotene, which has a variety of commercial uses (Ben-Amotz & Avron 1983). Since the description of the genus by Teodoresco (1905), much has been learned about *Dunaliella*. However, there are still gaps in our understanding of portions of the life cycle and the variety of growth habits of *Dunaliella*.

Isolated strains of *Dunaliella* spp. from soil and benthic samples exhibit a growth habit not widely reported for the genus which could be described as colonial, a perpetual palmelloid stage, or sarcinoid growth (Major et al. 2005, Kirkwood & Henley 2006, Henley et al. 2007, Buchheim et al. 2010). These isolates all originate from areas of highly variable environmental conditions unsuitable for most algae. Isolates from the

Great Salt Plains (GSP) in Oklahoma, USA experience extreme swings in temperature annually (-10 to > 50°C) and daily (as much as 30 degrees) as well as in salinity ranging from near freshwater to saturated brine or salt crusts. Those from Great Salt Lake (GSL) in Utah, USA are from the supralittoral zone that also likely exhibits large shifts in temperature and salinity with water level. As such, these organisms have been characterized as poikilotrophic, able to withstand extreme changes in environmental conditions (Major et al. 2005, Kirkwood & Henley 2006).

In order to deal with such sudden or prolonged periods of osmotic stress, *Dunaliella* can enter a palmelloid stage (Baas-Becking 1931, Watanabe 1983, Montoya & Olivera 1993, Leonardi & Cáceres 1997, Azúa-Bustos et al. 2010). The palmelloid stage is characterized by an expansion of the glycocalyx within which the cell undergoes morphological changes and eventually divides, resulting in mucilage dotted with cells (Watanabe 1983, Leonardi & Cáceres 1997, Borowitzka & Siva 2007). Ophir and Gutnick (1994) showed that mucilage significantly improves the survivability of desiccation for microorganisms, specifically *Escherichia coli*, *Erwinia stewartii*, and *Acinetobacter calcoaceticu*s strains with enlarged glycocalyces. Leonardi and Cáceres (1997) also suggest that the palmelloid stage is only formed during the sexually immature portion of the *Dunaliella salina* lifecycle. As will be shown, a palmelloid stage cannot fully explain the aggregation in our new isolates of *Dunaliella* (Major et al. 2005, Kirkwood & Henley 2006, Henley et al. 2007, Buchheim et al. 2010).

The fundamental goal of this study is to characterize and explain how two of these isolates of *Dunaliella* aggregate. Since an aggregating growth habit is not common among current *Dunaliella* isolates, it is also possible that this aggregation constitutes a novel growth habit for *Dunaliella*. It is known that *Dunaliella* may enter a palmelloid stage when exposed to stressors, but this novel aggregation may alter the understanding of the life cycle of the genus. Finally, for the sake of comparison and interpretation of the results of this study, a thorough literature review of intercellular adhesion and binding in the algal lineages Chlorophyta, Rhodophyta, and Ochrophyta is included here.

CHAPTER II

INTERCELLULAR ADHESION IN ALGAE

The eukaryotic algae are a polyphyletic, artificial construct that comprises diverse lineages responsible for a significant portion of the world's primary productivity. This grouping is spread across three kingdoms: Chromista, Plantae, and Protozoa. Organisms in these phyla represent a broad diversity in macroscopic and microscopic morphology and biochemistry. Species range from simple, microscopic single-cells to highly complex, multicellular structures. I will focus on photosynthetic members of Chlorophyta from Plantae as well as Rhodophyta and Ochrophyta from Chromista because these phyla represent a diversity of evolutionary history, morphology, and biochemistry which have been well studied. These lineages also exhibit a variety of means of intercellular adhesion, making them useful for such a review.

Surface Intermolecular Forces

The cell surface carries a negative charge due to the exposed phosphates of the phospholipid bilayer and the anionic glycoconjugates bound to most cells' exteriors which repel other cells due to both electrostatic interactions between the negative charges and adsorbed polar water molecules (Cowley et al. 1978). This intercellular repulsion

presents a problem for any intercellular adhesion. The most obvious solution to this problem is to neutralize the surface charge of cells. Doing so could prevent electrostatic repulsion and remove the hydration shell from around the cells, allowing Van der Waals forces to attract two microscopic cells together (Nir & Andersen 1977). It has been understood for some time that the neutralization of surface charge or bridging cationic charges are the mechanisms behind most chemical flocculants used for harvesting microalgae (Ries & Meyers 1968).

In natural systems, microalgae can consume all CO_2 from the surrounding liquid, raising the pH. At basic pH, divalent cations form chemical precipitates called mineral flocs that are large enough to bridge the surface charges of microscopic cells and cause the cells to flocculate (Sukenik & Shelef 1984). This phenomenon of flocculation at high pH is known as autoflocculation which is known to occur in Chlorophyta and Ochrophyta such as *Scenedesmus dimorphus* (Sukenik & Shelef 1984) and *Phaeodactylum trichornutum* (Spilling et al. 2011), respectively. Although autoflocculation has not been reported in Rhodophyta, the physical chemistry of the phenomenon should apply to all microorganisms.

For *S. dimorphus*, autoflocculation occurs at any pH > 8.5. However, this is also dependent upon the presence and concentration of specific divalent cations (Sukenik & Shelef 1984). Autoflocculation of *P. trichornutum* requires pH > 10 (Spilling et al. 2011). The mechanisms of autoflocculation are well understood and straightforward, but achieving both the necessary pH and specific divalent cations' concentrations is more complex. Both factors are directly affected by the biochemical activity of the algae in culture (Brady et al. 2014), so the specific conditions required to cause autoflocculation

are dependent upon a host of biotic and abiotic factors including the species present, nutrient conditions, and light levels.

Polymeric Adhesion

Most algal cells possess some form of extracellular polymeric substances (EPS) which are most commonly found in the form of a glycocalyx or cell wall (Hoagland et al. 1993, Martone et al. 2009, Mishra & Jha 2009, Michel et al. 2010, Popper & Tuohy 2010, Sørensen et al. 2011). Some lineages also have mineral deposits on the cell surface which form scales, thecae, or frustules (Eikrem & Throndsen 1990, Kröger & Poulsen 2008). Even in a simple model of cell surfaces increasing glycoconjugate content, the major component of EPS, led to increased adhesion for contacting cells (Nir & Andersen 1977).

The actual adhesive properties of glycoconjugates often depend upon physical principles including Van der Waals interactions and electrostatic charges (Hermansson 1999). By modifying the expression of glycoconjugates, the EPS properties can be altered to influence intercellular or cell-surface adhesion (Staats et al. 1999). These modifications can alter electrostatic charge in two key ways. By neutralizing electric charges, Van der Waals interactions can then adhere cells. By increasing opposing electric charges, electrostatic interactions can adhere cells. Furthermore, proteins secreted from the cell into the EPS can form chemical bonds, e.g., cross linking peptide chains between cells or acting as surface anchors for glycoconjugate adhesion.

Diatoms (Bacillariophyceae) are single celled ochrophytes which have silica frustules and produce a variety of EPS. These EPS are often used to adhere to substrates and other cells. Several types of adhesion have been described based on morphological

observations, but regardless of the type, diatoms adhere to substrates and each other by producing mucilage composed of polysaccharides. Cytochemical staining of adhered diatoms suggests that these polysaccharides are mainly anionic or acidic, and sulfation of polysaccharides may be important although it varies with species (Daniel et al. 1987). Increased proportions of acidic polysaccharides, specifically uronic acids, and sulphate groups increased when comparing adhered and unadhered diatoms. Adhered cells of the diatoms *Cylindrotheca closterium* and *Navicula salinarum* respectively increase glucose content by 59.6 % and 43.5 % in polysaccharides in order to adhere (Staats et al. 1999).

In the rhodophytes, spore adhesion is widely studied because adhesion is required for germination (Ouriques & Bouzon 2003). Tetraspores of *Champia parvula* attach to surfaces using a mucilage similar to the one discussed in ochrophytes. This attachment is dependent upon proteins, likely glycoproteins, and sulfphated polysaccharides (Apple & Harlin 1995). Spores of *Porphyra spiralis* adhere through a similar means (Ouriques et al. 2012). A study of thirty-one rhodophyte taxa showed that all but one possessed extracellular mucilage (Sheath & Cole 1990)

Chlorophyta is a diverse lineage with many aggregating species. *Chlorella pyrenoidosa* can be induced to aggregate if excess photosynthate is converted into bound and soluble polysaccharides (Yang et al. 2010). *Dunaliella salina* var. *palmelloides* forms large aggregates of cells inside an expanded glycocalyx. Although the cells within this mucilage originate from a single cell by cell division, these aggregates can adhere together to form even larger aggregates (Montoya & Olivera 1993). Although *Ulva* is known to adhere to substrates, the mechanism of the adhesion is unknown. Studies of

mRNA expression, however, show that *U. linza* possess homologs of thirty-nine adhesion or cell wall proteins from other species (Stanley et al. 2005).

Bioflocculation occurs when one species adheres to another causing flocculation of both.This interspecies adhesion has been reported in natural and laboratory studies. *Ettlia texensis* and *Chlorella vulgaris*, both chlorophytes, can be co-cultured to cause flocculation of both cells (Salim et al. 2014). Salim et al. (2011) showed that this also works for a variety of other chlorophyte species. Ben-Amotz used the diatom *Skeletonema* sp., an ochrophyte, to bioflocculate *Nannochloropsis* sp., a chlorophyte (Schenk et al. 2008). It appears that natural aggregation or the ability to autoflocculate is all that is required for one species to flocculate another.

Protein Binding

Lectins, or sometimes less specifically agglutinins, are common proteins in algal lineages (Hori et al. 1988, Hori et al. 1990). These highly specific, saccharide binding proteins are responsible for zygote recognition, binding, and fusion in the chlorophyte *Chlamydomonas* (Goodenough et al. 2007). Lectins play a similar role in the rhodophyte *Antithamnion* (Kim & Fritz 1993, Kim et al. 1996) and ochrophyte *Fucus serratus* (Bolwell et al. 1979). Generally, lectins also play roles in cell-cell recognition and adhesion (Sharon & Lis 1989). Since lectins have a high specificity, their binding and the resulting cell adhesion is often for intraspecific cell-cell adhesion.

Multicellularity

Multicellular organisms can be found in many distinct lineages. The evolution of mulicellular organisms from ancestral single celled organisms was independent in each of

these lineages. Regardless of the lineage, cells are adhered together by EPS in the form of cell walls or glycocalyces, so adjacent cells and their organization are determined at division. Rhodophyta, Ochrophyta, and Chlorophyta all have evolved multicellular growth forms. These three groups of algae share common traits but are also distinct in how the cells in these multicellular organisms adhere.

Cell walls are often considered the defining component of plants, but they are prevalent in algae as well. Cell walls adhere cells together because the chemical components of the wall are secreted by both daughter cells and bound to both plasma membranes. Plant cell walls also possess pectins, which are acidic polysaccharides rich in uronic acids. These are the same acidified polysaccharides that often make algal mucilage adhesive (Daniel et al. 1987). Unlike mucilage produced by algae, cell walls can be composed of neutral saccharides (Blumreisinger et al. 1983). Ochrophyte cell walls contain cellulose, similar to plants, but the majority of their cell wall is composed of anionic polysaccharides (Cronshaw et al. 1958, Kloareg & Quatrano 1988). Algal cell walls also possess uronic acids (Cronshaw et al. 1958). The rhodophyte *Calliarthron cheilosporioides* possess lignin, which is considered one of the key traits evolved by plants that allowed them to move from aquatic to terrestrial ecosystems (Martone et al. 2009). *Chlamydomonas reinhardii*, a commonly studied green microalga, has a cell wall composed entirely of glycoproteins. The arrangement and construction of these glycoproteins is shared by all other members of Volvocales (Roberts et al. 1985).

Intercellular transport is a necessity for multicellular algae. In addition to possessing cell walls similar to plants, multicellular algae also possess a continuous protoplast via plasmodesmata or pits. Similar to secondary plasmodesmata in plants, these

plasmodesmata form after the cell wall between dividing cells fully forms. Algae do not form plasmodesmata at cell division, like plant primary plasmodesmata. These connections have been reported without desmotubules in chlorophytes and ochrophytes (Bisalputra 1966, Franceschi et al. 1994). Members of Rhodophyta possess pits and pit plugs rather than plasmodesmata. The pit plug is composed of two parts. The endoplasmic reticulum captured within the cell wall between cells forms the pit core. The pit core is covered by the plasma membranes from the two cells, the pit cap (Ueki et al. 2008). Pits and pit plugs differ from plasmodesmata because there is no symplastic connection.

When comparing the adhesion of multicellular to single celled algae, the most striking difference is the origin of the cells' arrangements. In multicellular organisms, the arrangement of cells is determined at division. Since single cells' adhesiveness can be regulated, they are able to rearrange their organization if necessary . Single cells can often regulate their adhesiveness by modulating the acidification of polysaccharides by incorporating more uronic acids (Staats et al. 1999). Uronic acids provide adhesiveness to plant cells in the form of pectins and they can also be found in the cell walls of multicellular algae (Daniel et al. 1987). It seems that the diversity of ways in which algae cells adhere to each other is conserved from single cells through the lineages to more complex, multicellular organisms.

CHAPTER III

METHODOLOGY

General Maintenance of Cultures

Four isolates from Great Salt Lake, Utah (GSL) were selected for examination in all experiments. Isolates GSL-3A4 and GSL-3C2 were obtained from sediments. These isolates have exhibited an aggregating growth habit since isolation. GSL-6/1 and GSL-12A4 were obtained from the near shore plankton and have exhibited the more familiar single cell growth habit of *Dunaliella* since isolation (Henley, unpublished). Additional information about the isolates can be found in Table 1. All samples were originally inoculated from archival liquid cultures into 75 mL of modified 10 % (w/v) NaCl AS-100 media in sterile 125 mL Erlenmeyer flasks stoppered with sterile cheesecloth or foam plugs (Henley et al. 2002). Since isolation, cultures have been maintained in this media in a Percival incubator at 18-22 °C and ~25 µmol photons/m^2/sec.

Cultures for the present experiments were kept in a climate controlled growth room between 24 °C and 30 °C under 1000 W metal halide lamps (Plantmax PX-MS1000) which provided 200 µmol photons/m^2/sec as measured at the surface on which culture containers were kept using a LI-Cor LI-189 cosine sensor. Secondary cultures were also

maintained in screw top glass test tubes containing 10-20 mL of media in both the growth room and the Percival incubator in case of contamination of primary cultures. All cultures were maintained by weekly or monthly transfers of 1 mL of inocula from old cultures into fresh media for experimental and secondary cultures respectively.

Table 1. Descriptions and isolation information about the isolates used for all experiments. All isolates were from samples taken in mid-May, 2008.

Isolate	**Growth Habit**	**Sample Type**	**Approximate Sample Location**	**Isolation Date**
GSL-3A4	Aggregating	Benthic	41° 26’ N 112° 40’ W	07/16/2008
GSL-3C2	Aggregating	Benthic	41° 26’ N 112° 40’ W	07/08/2008
GSL-6/1	Unicellular	Planktonic	41° 26’ N 112° 40’ W	07/08/2008
GSL-12A4	Unicellular	Planktonic	40° 57’ N 112° 12’ W	07/09/2008

Microscopy

1 mL samples from visibly dense cultures were collected and fixed in 4 % formaldehyde. Cells were then pelleted by centrifuging at 500 g for 5 min and washed with deionized (DI) water three times. Samples were then either resuspended in 1 mL of DI water or

stained with 1 mL 0.05 % (w/v) Alcian Blue (pH 3.00) for 1 hr. Alcian Blue is a cationic stain often used for examining glycocalyces or other glycoconjugates (Scott et al. 1964). After staining, cells were again pelleted and washed three times with DI water before resuspension. Unstained samples of aggregating strains were also relief stained with a drop of India ink on the slide. Any expansion of the glycocalyx appears as a cleared area around cells against the background of India ink (Duguid 1951). All samples were examined as wet mounts using Nomarski microscopy at 400-1000X total magnification on a Nikon Eclipse 80*i* or Nikon Eclipse N*i*.

Throughout the duration of this work and for all experiments, microscopy was crucial for understanding what was occurring within cultures. Because of this, each sample was typically studied by examining the entire area of the coverslip. Samples were used for making multiple slides. Cultures were commonly resampled to ensure consistency of observations. Selected micrographs have been presented in the results to show the observed trends for cultures, but these do not adequately represent the full extent of the microscopy which was undertaken.

Co-Culture and Filtered Media

Samples of each aggregating isolate were co-cultured with each unicellular isolate to determine if this alters the growth habits of isolates. Cultures were inoculated with 1 mL each of an aggregating and a single cell strain for a total of four co-cultures as shown in Table 2. These were then observed on a weekly basis visually and under a microscope for qualitative changes in growth habit and composition, e.g., cell morphology and relative proportion of individual versus aggregated cells compared to single isolate cultures.

Cultures were also transferred into fresh media on a weekly basis. These co-cultures were maintained for a total of seven weeks.

Table 2. Pairwise co-culture combinations.

Isolates in Co-Culture	
GSL-3A4	GSL-6/1
GSL-3A4	GSL-12A4
GSL-3C2	GSL-6/1
GSL-3C2	GSL-12A4

Active cultures of GSL-6/1 and GSL-12A4 were divided into two flasks each, resulting in ~37 mL of culture. Similar cultures of GSL-3A4 and GSL-3C2 were filtered through glass microfiber filters (Whatman GF/F) to remove cells but allow any water soluble factors in the conditioned media to remain. These conditioned media were then divided and added back to the cultures of GSL-6/1 and GSL-12A4 as shown in Table 3, resulting in cultures of ~75 mL. These were then allowed to grow for three weeks, and microscopy observations were conducted weekly.

Table 3. Pairwise addition of conditioned media to active cultures.

Culture	Media From
GSL-12A4	GSL-3C2
GSL-12A4	GSL-3A4
GSL-6/1	GSL-3C2
GSL-6/1	GSL-3A4

Lectin Inhibition

Algae are known to produce lectins which can bind to cell surface polysaccharides and cause aggregation (Chu et al. 2007). Monosaccharides can inhibit the binding activity of lectins. In order to test the effects of monosaccharides on aggregation in GSL-3A4 and GSL-3C2, galactose, mannose, fucose, N-acetyl-glucosamine, and N-acetyl-galactosamine, all monosaccharides known to inhibit lectin binding, were added individually to existing cultures and at inoculation of new cultures. Each monosaccharide was added to 1 mL of established culture in a 7 mL scintillation vial to a final concentration of 2 mM.

Additionally, a 7 mL scintillation vial containing medium was inoculated with a 17 µL sample from established cultures. This medium contained a monosaccharide at 2 mM final concentration in 1 mL final volume. All treatments were then maintained for three weeks, and microscopy observations were made weekly.

Divalent Cation Removal

Divalent cations are necessary cofactors for ligand binding by many proteins as well as for autoflocculation. In order to assess if divalent cations are required for aggregation in GSL-3A4 and GSL-3C2, cultures were grown without added Mg^{2+} or Ca^{2+}, the major divalent cations in AS-100. Four different media were created: modified AS-100 (Control), modified AS-100 with no added Mg^{2+} (-Mg), modified AS-100 with no added Ca^{2+} (-Ca), and modified AS-100 with no added Mg^{2+} nor Ca^{2+} (-Mg -Ca). We used ten replicate cultures for each combination of isolate and media.

To quantitatively measure any effects on aggregation, 5 mL samples from the cultures were filtered through mesh with a nominal pore size of 35 µm. This is sufficiently large to allow two attached cells, such as dividing cells, to pass through but small enough to retain larger aggregates. The filtrate which passed through the mesh was then filtered through a glass microfiber filter (Whatman 934-AH). The retentate from the mesh was then washed off of the mesh using 10 % (w/v) NaCl and filtered through a glass microfiber filter. Filters were placed into 15 mL conical bottom centrifuge tubes and chlorophyll was extracted overnight using 3 mL of 90 % (v/v) acetone saturated with $MgCO_3$. Chlorophyll extracts were evaluated using a Turner Aquafluor handheld fluorometer. Aggregation efficiency was defined as $100\ \% \times \frac{chlorophyll\ from\ retentate}{chlorophyll\ from\ retentate + filtrate}$.

CHAPTER IV

FINDINGS

Microscopy

Isolate GSL-3A4 exhibits aggregates where individual cells are trapped within an expanded glycocalyx. This is most apparent in Figures 1C and D where the mucilage has been stained with Alcian Blue. Further support for this can be seen in Figures 1E and F. The India ink is fully excluded from the center of the aggregates. Because of the expanded glycocalyx, the pigment particles in the ink cannot penetrate the interior of aggregates. The distinct individual aggregates can be seen clearly in four portions of Figure1: C, D, E, and F. Without a contrasting stain, it is more difficult to distinguish the individual aggregates (Figure 1A and B). Individual cells within aggregates exhibit varied cell shape, but they are generally more rounded than non-aggregated cells found in culture (Figure 1G).

Individual cells can be found in culture; these cells are similar in size to GSL-12A4 (Figures 1G and 3). They often have distinct pyrenoids and a transparent anterior region. Their flagella are noticeably shorter than isolates which grow only as individual cells.

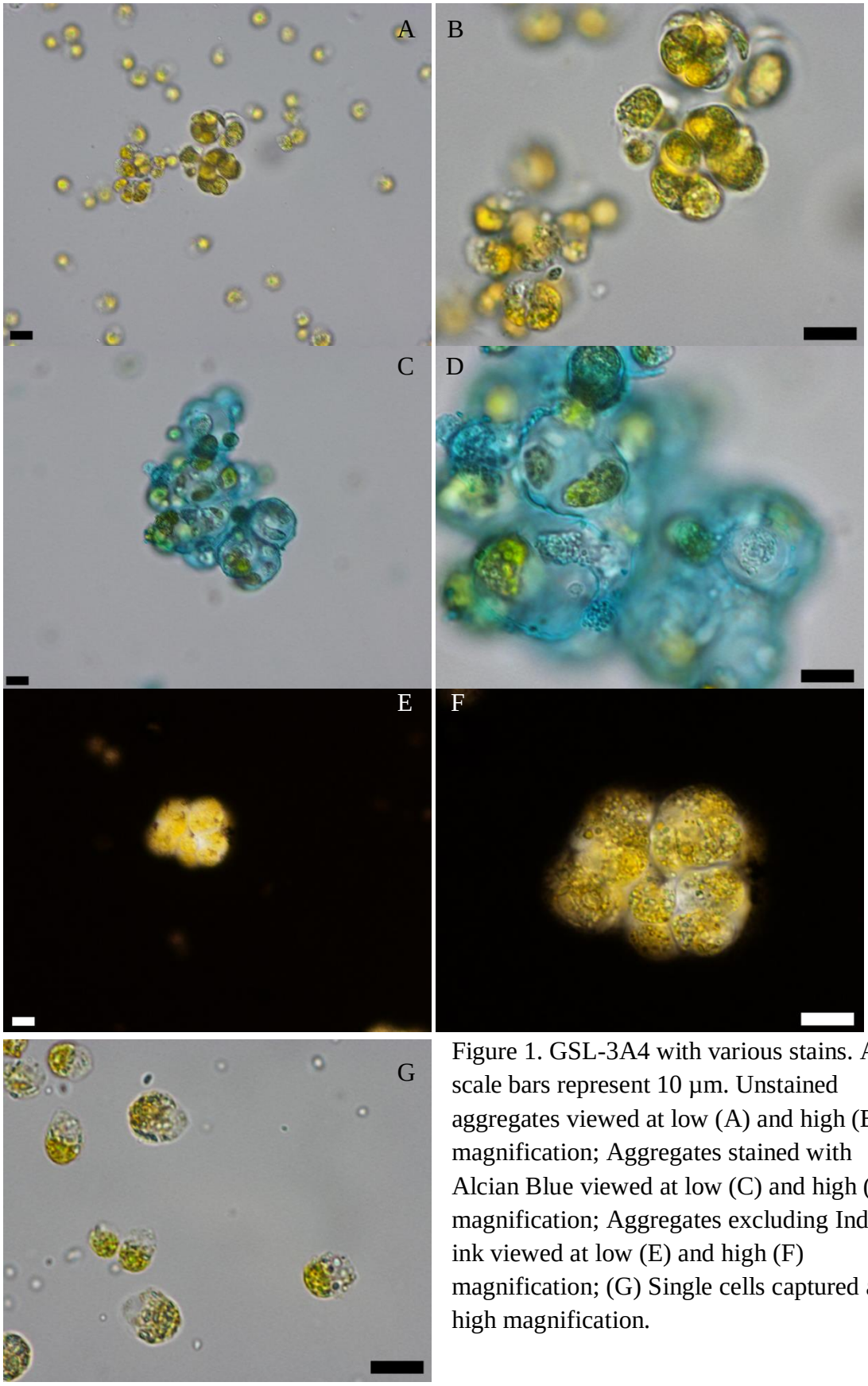


Figure 1. GSL-3A4 with various stains. All scale bars represent 10 µm. Unstained aggregates viewed at low (A) and high (B) magnification; Aggregates stained with Alcian Blue viewed at low (C) and high (D) magnification; Aggregates excluding India ink viewed at low (E) and high (F) magnification; (G) Single cells captured at high magnification.

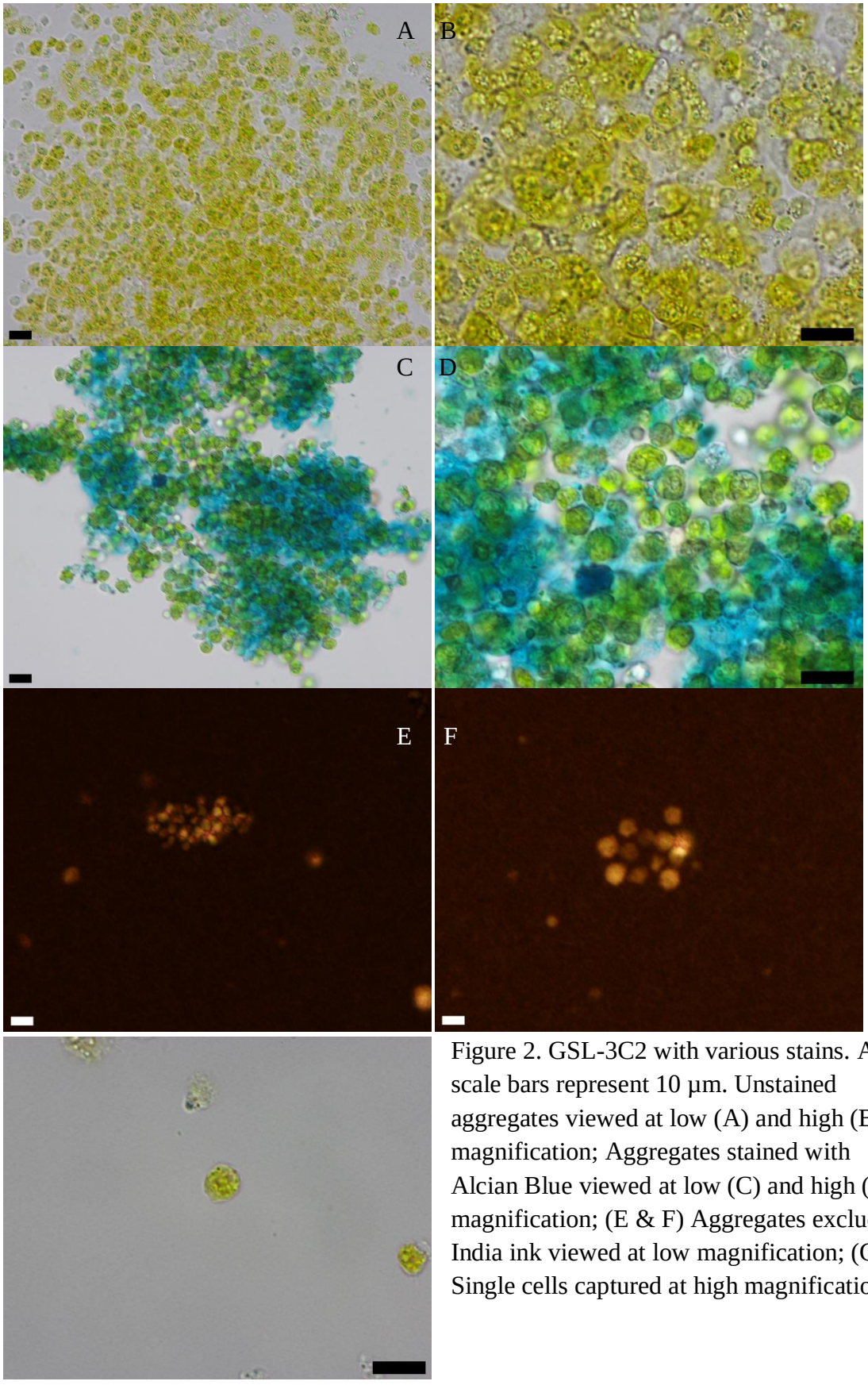


Figure 2. GSL-3C2 with various stains. All scale bars represent 10 µm. Unstained aggregates viewed at low (A) and high (B) magnification; Aggregates stained with Alcian Blue viewed at low (C) and high (D) magnification; (E & F) Aggregates excluding India ink viewed at low magnification; (G) Single cells captured at high magnification.

Isolate GSL-3C2 exhibits a more complex aggregating behavior than GSL-3A4. Aggregates similar to those produced by GSL-3A4 can be found, but such aggregates appear to be a transient state and form a minority of the aggregates present throughout the life of a culture. Nearly all aggregates in GSL-3C2 have a distinct growth pattern in aggregates. This growth form is most apparent in Figures 2A and B. The cells were compacted together, and their shape is dependent upon their contact with adjacent cells. This is not accompanied by an expansion of the glycocalyx, as clearing can be seen when stained with Alcian Blue (Figures 2C and D). Likewise, India ink can penetrate much more extensively between the individual cells in such aggregates, as is shown Figure 2E and F.

Cells which are not aggregated in culture appear as spheres, often similar to those present at the edges of aggregates or in areas of aggregates with clearing, such as where cells have not enlarged to meet compact against other cells. These individual cells often have a pointed anterior at which the flagella are attached. They have a distinct pyrenoid as well as stigma (Figure 2G).

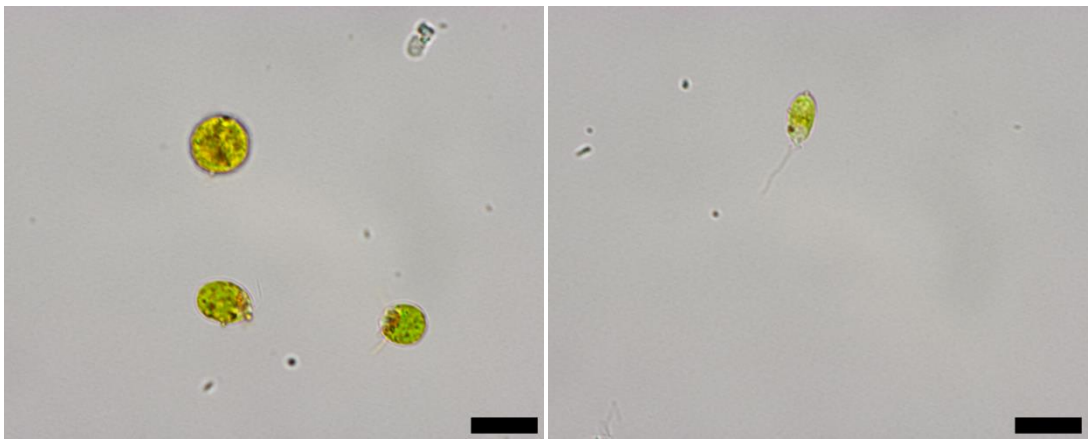


Figure 3. Single cells of GSL-12A4 with scale bars representing 10 µm.

Isolate GSL-12A4 (Figure 3) is always present as distinct, individual cells. Although specific cell shape can vary with culture age, typically growing laterally, cell length is consistent. Despite the presence of a glycocalyx, the cells do not aggregate. Flocculation can occur when there are sufficient dead cells or mineral particulates in the culture for live cells to adhere to.

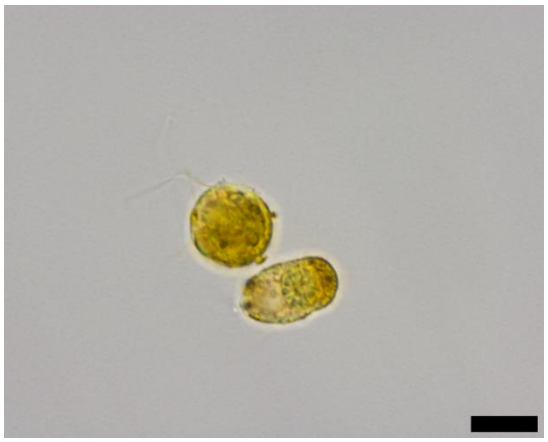


Figure 4. Single cells of GSL-6/1 with a scale bar representing 10 µm.

GSL-6/1 is noticeably larger than the other isolates used here. Individual cells tend to be nearly spherical or elongate. They often also have a pronounced pyrenoid surrounded by an amylosphere. This is also the only strain to appreciably accumulate carotenoids as the cultures age, producing the golden coloration visible in Figure 4 compared to the other isolates. Much like GSL-12A4, cells of GSL-6/1 have a glycocalyx, but they do not aggregate. Again, cultures containing sufficient dead cells or mineral particulates may flocculate.

Co-Culture and Filtered Media

When aggregating isolates and isolates which grow as single cells were cultured together, morphological distinctions could be made such as GSL-6/1 being double the size of the other isolates. Likewise, the morphology of cells within aggregates from

GSL-3A4 and GSL-3C2 were unique compared to individual cells. The relative abundances of individual cells and aggregated cells remained similar within co-cultures for seven weeks of the experiment. There were also no observable trapped cells, i.e., individual cells lodged within the aggregated cells. Individual cells could commonly be found on the surface of aggregates. These individual cells maintained their own morphology, distinct from the cells within aggregates.

When media from established aggregating cultures was filtered and added to established cultures of non-aggregating isolates, cell morphologies did not change (Figure 5). GSL-12A4 and GSL-6/1 displayed the same morphological characteristics as under normal media conditions. Flocculation occurred when living cells were attached to dead cells due to culture age.

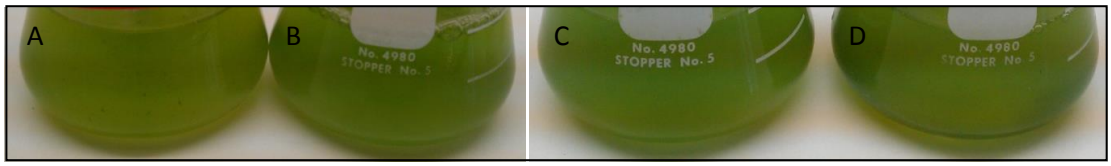


Figure 5. Individual cell cultures with media filtered from aggregating strain cultures. (A) GSL-12A4 culture with media from GSL-3A4; (B) GSL-6/1 culture with media from GSL-3A4; (C) GSL-6/1 culture with media from GSL-3C2; (D) GSL-12A4 culture with GSL-3C2 media.

Lectin Inhibition

When monosaccharides were added to established cultures or to cultures at inoculation, there were no observable effects on cell aggregation. Morphological comparison to controls with no added compounds showed no appreciable difference; gross comparisons

can be made in Figure 6. The aggregation of isolates held for the entire three week duration of the experiment. Furthermore, there was no distinct increase in individual cells. Although a small proportion of individual cells are always present in aggregating isolates, they were not in excess when compared to control treatments.

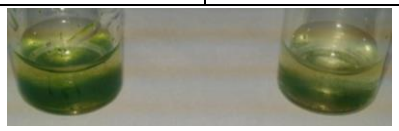
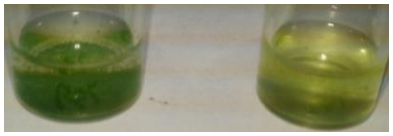
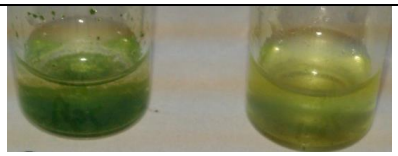
Monosaccharide	Control		Treatment	
	GSL-3C2	GSL-3A4	GSL-3C2	GSL-3A4
Galactose				
Mannose				
Fucose				
N-acetyl-glucosamine				
N-acetyl-galactosamine				

Figure 6. Various monosaccharide treatments applied to active cultures of the two aggregating isolates as well as corresponding control treatments.

Divalent Cation Removal

Preliminary data using a yeast protocol (Stratford & Carter 1993) showed that flocculation efficiency was lowest under the control treatment (Figure 7). There was a

significant difference between the treatments (ANOVA, $F_{3, 56}$=8.573, p<0.0001). Post hoc analysis with Tukey's HSD confirmed the apparent difference in Figure 7; the control was significantly different from all other treatments while all other treatments were not significantly different from each other, at an α=0.05 level. It was unexpected that treatments -Mg, -Ca, and -Mg -Ca yielded greater flocculation efficiencies than the control.

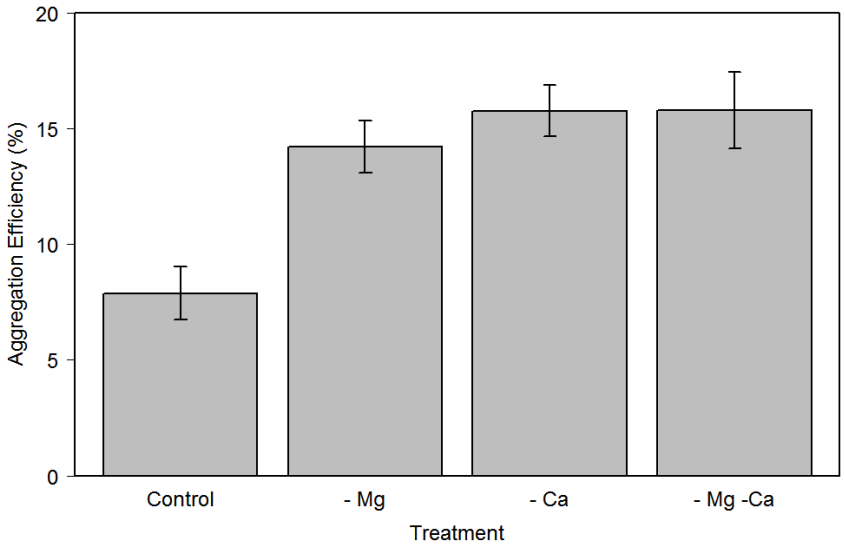


Figure 7. Flocculation efficiency of GSL-3A4 exposed to each of the four media treatments (Control: 7.89±1.15, n=15; -Mg: 14.22±1.14, n=15; -Ca: 15.76±1.11, n=15; -Mg -Ca: 15.80±1.66, n=15).

After this initial experiment, replication utilizing the final method, as previously described, faced repeated culture crashes. In all cultures, cells inoculated into experimental flasks would not grow beyond the inoculation density. These cells lost pigmentation, flocculated, and settled to the bottom of the flasks. We used microscopy to determine that these cells died, probably because of the use of new foam stoppers.

Returning to secondary cultures allowed for the experiment to resume. Figure 8 shows the results of all four isolates exposed to the control and -Ca. For GSL-3A4, exposed to the control treatment, flocculation efficiency remained similar to the preliminary experiment (Figure 7) despite measuring chlorophyll content in aggregates and single cells rather than settling times, as is done with yeast. The treatment -Ca showed reduced flocculation efficiency when compared to the preliminary results (Figure 7). Extensive comparisons cannot be made because this change in quantitative data was corroborated by qualitative observations.

In general, cell morphologies in these experiments were inexplicably inconsistent with those previously described for GSL-3A4 and GSL-3C2. Cultures tended to have much higher proportions of individual cells, appearing similar to mixed cultures of aggregating and individual cell strains. Aggregates were still present with morphologies matching those originally described for their isolate. Because of these issues and time limitations, the experiment was discontinued without exposing isolates to either -Mg or -Mg -Ca treatments.

Two-factor ANOVA of the results shown in Figure 8 showed that isolates do not aggregate at significantly different efficiencies between treatments ($F_{1, 72}$=1.689, p=0.198). Similarly, isolates did not differ significantly within treatments ($F_{3, 72}$=1.766, p=0.161). The interaction between treatments and isolates also showed no significant differences ($F_{3, 72}$=0.120, p=0.948).

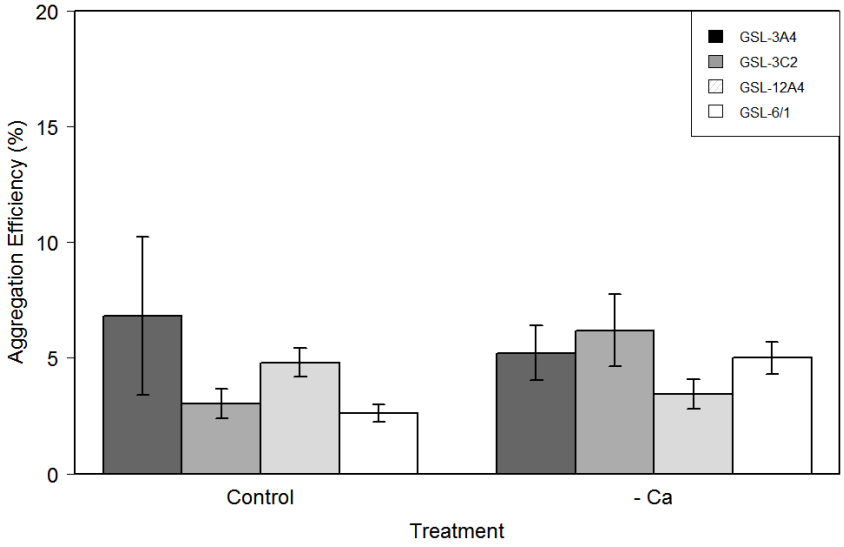


Figure 8. Average flocculation efficiencies with standard errors (n=10) of all four isolates exposed to two media treatments.

CHAPTER V

DISCUSSION

Comparing the morphologies of our four isolates between each other and descriptions of known species (Borowitzka and Siva 2007) allows for the characterization and identification of the strains. Isolate GSL-3A4 displays the main characteristics of a palmelloid stage as described by Leonardi and Cáceres (1997), with its large expanded glycocalyx dotted with rounded cells. Of the three species of *Dunaliella* which show a prominent palmelloid life stage, this isolate most resembles *D. viridis* var *palmelloides*. The individual cells' shapes distinguish it from the cylindrical *D. minuta* var *palmelloides*, and the source habitat distinguishes it from the subaerial *D. atacamensis*.

Despite its aggregation, isolate GSL-3C2 lacks the expanded glycocalyx characteristic of a palmelloid stage. Instead, GSL-3C2 exhibits a unique growth. Individual cells are commonly spherical with a basal, spherical pyrenoid. This spherical cell shape is characteristic of *D. minutissima*. Unfortunately, this species is only mentioned in literature to provide its initial description (Ruinen 1938), taxonomic reassignment (Massyuk 1973), and by Borowitzka and Siva (2007), making it difficult to confirm this identification.

The two individual cell isolates are more readily identified because of their more common growth habit. GSL-12A4 has a small cell size characteristic of the section Virides of *Dunaliella*. Because of its pyriform shape, clear anterior, and high salinity tolerance it is most likely *D. viridis*. This species has been reported in GSL since the early 1930s, although initially misidentified as *Chlamydomonas* (Flowers 1934). The larger cell size of GSL-6/1 places it outside the section Virides. The partial carotenoid production leading to light orange cells under high light in older cultures as well as the pronounced pyrenoid and amylosphere characterize the isolate as *D. parva*. (Borowitzka and Siva 2007). This species was common in the Dead Sea, but has not been reported for GSL (Oren & Shilo 1982). The fact that it can be prolific in other inland hypersaline water bodies means it is likely that this species would also be present in GSL.

GSL-3A4 and GSL-3C2 aggregate in different manners. GSL-3A4 forms the previously described palmelloid stage which is shown by the excess EPS visible in Figure 1B. Furthermore, the shortened flagella of individual cells of this isolate can be attributed to their regrowth after exiting the palmelloid stage. Since the flagella are often shed into the layers of expanded glycocalyx, after cells break free of the EPS, they must regrow their flagella resulting in their shorter length compared to cells which have never entered the palmelloid stage (Borowitzka & Siva 2007). In contrast, GSL-3C2 exhibits a distinct growth habit. Both isolates were obtained from substrate samples and are similar to other passing observations reported of algal cells in a palmelloid stage or forming multicellular coating on substrates at GSL (Brock 1975). Studies of substrate borne *Dunaliella* have only been parts of larger collection or survey efforts with little other research (Watanabe 1983, Arif 1992, Kirkwood & Henley 2006, Sathasivam et al. 2012).

The growth pattern of GSL-3C2 suggests that this isolate modulates EPS composition to induce adhesion. Previously, this growth habit has only been described in these and other isolates of *Dunaliella* by Buchheim et al. (2010) from a biological survey. Sarcinoid genera are typically lumped together as a group, but the relationship of these genera to each other and others is unclear. Watanabe et al. (2006) showed that one group of these organisms is sister to *Dunaliella*. Buchheim et al. (2010) also showed that an isolate of *Chlorosarcinopsis gelatinosa* (CCMP 1511), a sarcinoid algae, is actually a *Dunaliella* sp. Soil isolates of *Dunaliella* included in the Buchheim et al. (2010) phylogeny suggest that they may be distinct species and that the genus may require taxonomic rearrangement, possibly incorporating other sarcinoid alga. Further studies of *Dunaliella* isolated from soil or substrates may show that an aggregating growth habit is more prevalent in *Dunaliella* than is currently recognized. Isolates have been obtained but not widely studied from salt flats and desert gypsum crusts, such as strains FL-1 and BSF-1, 2, and 3 (Buchheim et al. 2010). Discoveries of other isolates or species in such habitats may lead to rearrangement or additions to the genus.

Species of *Dunaliella* produce a variety of carbon rich products from photosynthesis (Craigie & McLachlan 1964, Fabregas et al. 1989, Giordano & Bowes 1997). They can also be induced to form an adhesive palmelloid stage under certain environmental conditions (Lerche 1937). However, the palmelloid stage involves division within an expanded glycocalyx, resulting in multiple cells captured within mucilage. Multiple palmelloid stages may then adhere together forming larger aggregates. To date, there have been no descriptions of individual cells of *Dunaliella* adhering together to form aggregates in the manner isolate GSL-3C2 does. Since GSL-3C2 was isolated from the

benthos near shore, it is possible that this aggregating behavior increases desiccation survivability as water levels change. It has been shown that *Dunaliella* in a palmelloid stage are better equipped to survive desiccation (Henley et al. 2007) and possibly freshwater exposure, as with GSP 109-1 and 112-2 (Kirkwood and Henley 2006). The composition of *D. salina*'s EPS has been studied by Mishra et al. (2011). Comparison of the EPS of individual and aggregate cells of GSL-3C2 and other isolates of *D. minutissima* will inform our understanding of how GSL-3C2 is aggregating. Much is already known about the various means *Dunaliella* uses to combat osmotic stress, but aggregating growth and modification of EPS have not been thoroughly examined as a mechanism by which species may cope with such stress.

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VITA

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