CHARACTERIZATION OF A UNICELLULAR COCCOID GREEN ALGA COLLECTED FROM THE SALT PLAINS NATIONAL WILDLIFE REFUGE, OKLAHOMA

By

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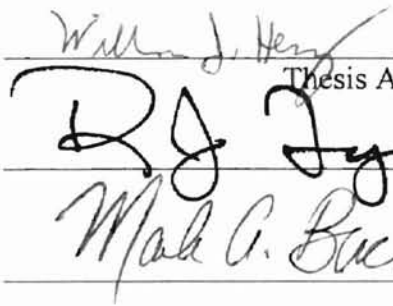

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PREFACE

An unusual, small (2 μm diameter), coccoid, unicellular, green alga was isolated from a hypersaline pond at the Salt Plains National Wildlife Refuge in Oklahoma, USA and given the designation 980625-4A. This alga shows remarkable ability to tolerate a wide range of salinities (0-140 g-L^{-1} NaCl) and temperatures (0-40 °C). Efforts to identify this alga were based upon measurements of cell size, ultrastructure, and modes of cell division using electron microscopy, HPLC analysis of pigments, and cell wall staining with ruthenium red. The alga exhibits a smooth outer cell wall with no evidence of outer or vestigial structures. Transmission electron micrographs revealed one cup-shaped chloroplast, one mitochondrion, one nucleus with one nucleolus. Several oval starch grains are interspersed between stacks of thylakoid membranes in the chloroplast. An accumulation body is associated with the mitochondrion. TEM micrographs show no evidence of pyrenoids. Cell division occurs by autosporulation. Only two autospores are produced from each mother cell. Ruthenium red minimally stains the cell wall of 980625-4A, but significantly stained three *Chlorella* species. Pigments present are chlorophylls *a* and *b*, and major carotenoids are lutein, β-carotene, violaxanthin, neoxanthin, and vaucheriaxanthin ester. Pigment profiles exhibited greater similarity to pigments from *Nannochloris* sp. UTEX 2378 than other examined algae. These findings suggest 980625-4A is closely related to the genus *Nannochloris*. This is supported by recent 18S rDNA sequencing. Specific growth rates were measured at 25 and 40 °C in

AS100 media with salinities ranging from 0-120 g-L^{-1} NaCl. Growth rates showed statistically significant relationships to salinity, temperature, and combined effects of salinity and temperature. Growth rates at 25 °C showed minor changes over the range of salinities. Growth rates were lower at 40 °C for all salinities, and cells showed no growth at 40 °C and 0 g-L^{-1} NaCl. Ultrastructural distortions were observed in cells grown at 40 °C at all salinities. Osmolytes identified by HPLC-mass spectrometry include proline, glycerol, glucose, and glucosylglycerol. A comparison of cells grown at 0 and 40 g-L^{-1} NaCl showed a 1329 % or 13-fold increase in proline concentrations. Elevated salinity appears to protect 980625-4A from thermal stress if sufficient energy is available to sustain osmolyte production.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

Figure Page

INTRODUCTION

Extreme environments are found throughout the world and support a wide variety of unusually adapted organisms. Extreme environments have been defined as environments where most organisms cannot survive (Kushner 1993), such as high temperature (to 150 °C), cold temperature (as low as –16 °C) (Arrigo and Sulivan 1992, Gorbushina and Krumbien 1999, Russell and Hamanoto 1998), high salinity (to 6.9 M NaCl, Javor 1989), high (11.6) or low (1) pH, high concentrations (to 10^{-5}-10^{-4} M) of toxic metals, such as As, Cd, Cr, Cu, Hg, the presence of organic solvents (Kushner 1993), and pressures encountered at depths almost three kilometers below the surface of the earth (Kerr 1997).

A. Hypersaline environments

Some of the more interesting extreme environments are hypersaline. The continental United States of America contains numerous, widely scattered hypersaline habitats, including the Great Salt Lake and Bonneville Salt Flats in Utah, ancient ocean beds in New York, New Mexico, Kansas, Texas and Oklahoma, and salt domes in Louisiana. Hypersaline microbial communities are generally composed of Archaea, Eubacteria, heterotrophic and autotrophic sulfur bacteria, cyanobacteria, microalgae, diatoms and the flagellated chlorophyte *Dunaliella* Teodoresco 1905.

Microorganisms are classified according to the salinity ranges in which they survive: halotolerant (non-halophile which can tolerate salt), non-halophile (less than 0.2 M NaCl), slight-halophile (0.2-0.5 M NaCl), moderate-halophile (0.5-2.5 M NaCl), borderline extreme halophile (1.5-4.0 M NaCl), and extreme halophile (2.5-5.2 M NaCl),

(Kushner 1978). Haloversatile designates organisms capable of living in zero to salt saturated environments with an optimum growth rate in the presence of salt (James et al. 1990). Euryhaline denotes organisms that live in a wide range of salinities.

B. The Salt Plains National Wildlife Refuge

The Salt Plains National Wildlife Refuge (SPNWR) (36 °N, 98 °W), Oklahoma, a hypersaline extreme environment, was the study site for this thesis work (Figs. 1, 2). The salt flats of the SPNWR consist of 4,451 hectares (11,000 acres). The surface of these salt flats changes with weather events. Water runoff from the west, north, east boundries creates new streams and wash areas by erosion and deposition. The salt source is an ancient seabed located in the aquifer. Groundwater seeps through the ancient seabed dissolving salts, and where it surfaces, a salt crust is formed as evaporation occurs. Ephemeral evaporative ponds, having a surface area of one or two to many square meters, dot the saline landscape throughout the year. They range in salinity from near 0 M NaCl to about saturation at 5.2 M NaCl. A layer of salt crystals, from one to approximately 500 mm thick, forms a crust on much of the surface of the sandy soil except following heavy rainfall.

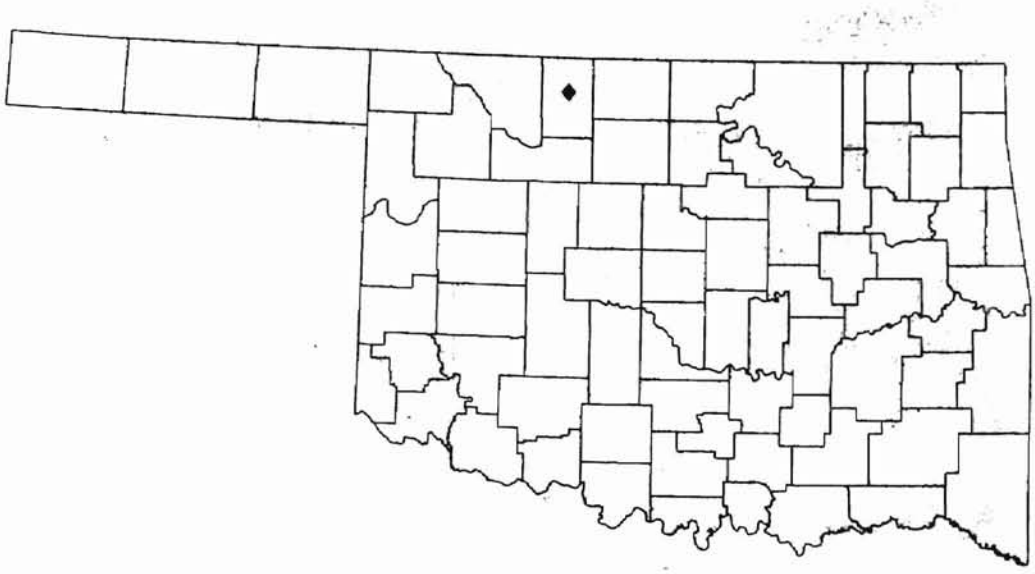

Figure 1. Map of the State of Oklahoma, USA. (♦) is the location of the Salt Plains National Wildlife Refuge.

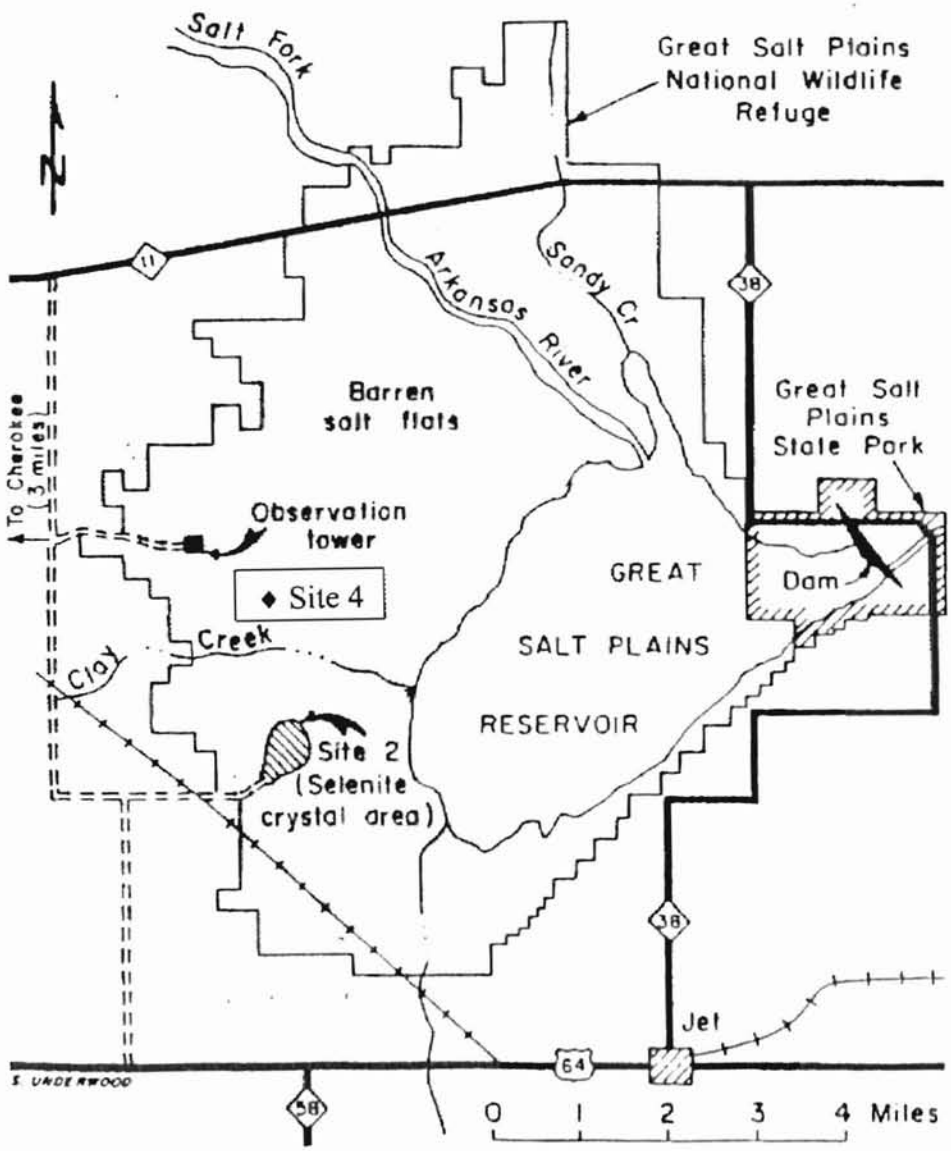

Figure 2. Salt Plains National Wildlife Refuge, Alfalfa County, Oklahoma, USA. (♦) is the location where 980625-4A was collected.

Weather plays an important role in shaping the sandy surface, ephemeral ponds and streams at the salt flats. During drought months, the salt crust may increase in thickness from 1 to approximately 2 cm and salt nodules may form in salt saturated ponds. During rainy periods, the salt layer is partially dissolved, leaving exposed sand. Extremes of high temperature are reached most often in July, August, and September, with typical daily maximum air temperatures of 35 to 42 °C. Daily temperature minima range from 17 to 25 °C. Average monthly rainfall for these months totals 15.7 cm. December, January and February tend to be the coldest months, with air temperatures from daily maxima of –5 to 20 °C and daily minima of –15 to 5 °C. Average monthly rainfall ranges from 1.8 to 9.3 cm (MESONET Data 1998-2000).

The three major sources of nutrients at the salt plains include hundreds of thousands of migrating birds, surface streams and runoff. During rain events, water runoff containing dissolved fertilizers and animal wastes from neighboring farmland and cattle pastures enter the salt plains on the west, north and north-east boundaries. Nutrients are also brought to the surface with seepage from underground saline water.

The environmental extremes of the ephemeral ponds and streams at the SPNWR have not been extensively studied. To explore the range of environmental changes experienced by aquatic organisms over a summer day, a field survey of an ephemeral pond at the north boundry of the SPNWR was conducted during August 6, 1998. Salinity was measured at 52.7 $g\text{-}L^{-1}$ total dissolved solids, and hourly measurements were taken from 06:30 (sun-rise) to 16:30 (late afternoon) when temperature readings were essentially constant for three consecutive readings (Fig. 3). Measurements were made of the air and water temperature, pH, dissolved O_2 concentrations, photon flux density

(PFD), and oxidation-reduction potential (ORP). Air and water temperatures, pH, and dissolved oxygen increased steadily throughout the day. The water temperature increased from 16 °C at dawn to 32 °C at mid afternoon. During this time the pH increased from 9.1 to 9.7 and dissolved O_2 increased from 0 to 20 M. The photon flux density above the pond ranged from 9.5 to 1710 μmol photons $\cdot m^{-2} \cdot s^{-1}$, and ORP ranged from –61 to +114 mV measured against an Ag/AgCl reference electrode.

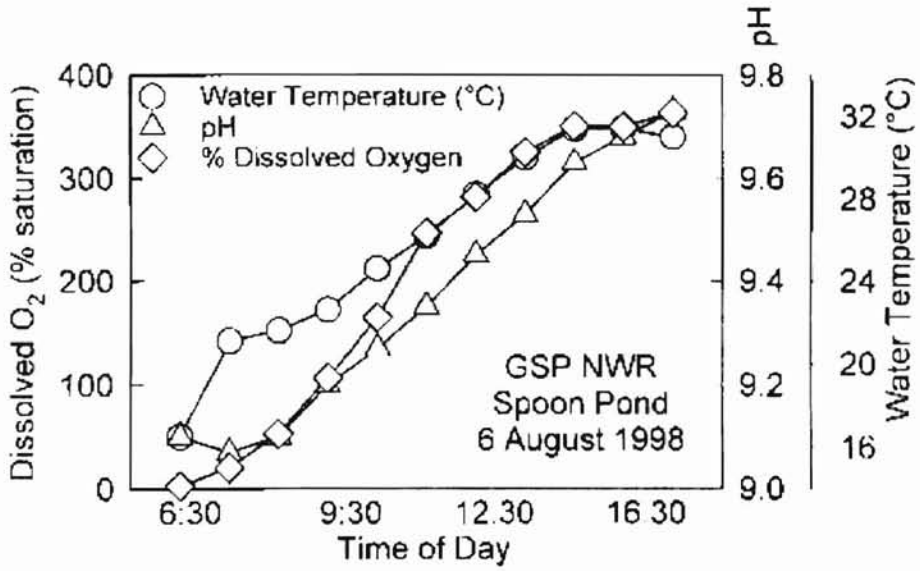

Figure 3. Water temperature, pH, and dissolved oxygen levels measured at a saline pond at the SPNWR on August 6, 1998

Despite the harsh environment and great fluctuations in weather at the SPNWR, microbial life is widely dispersed and found throughout the year on the salt flats (personal observations). An anoxic layer of black sand was found 2 mm beneath the surface of the sandy soil around and beneath the pond. The air around the ephemeral pond smelled of sulfurous compounds such as H_2S, which were most likely produced by sulfate-reducing

microorganisms commonly found in anoxic environments. Benthic and planktonic organisms were collected from the pond and examined in the laboratory. The benthic species observed were three *Oscillatoria*-like spp., a *Spirulina* sp., an amoeba-like unicellular organism, and four distinct species of diatoms. The planktonic organisms collected were unicellular green algae of different sizes, both flagellated and non-flagellated.

Algae and cyanobacteria were found in ephemeral ponds and streams elsewhere in the SPNWR on patches of damp sandy soil and in other areas 2 mm below the surface. In these areas of the salt flats different organisms were collected: *Vibrio* sp., *Nostoc* sp., *Anabaena* sp., *Vorticella* sp., *Zoothamnium* sp., a possible *Tetrahymena* sp., and *Dunaliella* sp. Other unidentified green unicellular algae were also isolated from the SPNWR. The identification of one of these, a small (~2 μm), coccoid, green alga (980625-4A), is described in greater detail in later sections of this thesis. This alga has remarkable adaptational abilities: it can grow in fresh water and artificial sea water medium in salinities to 120 $g\text{-}L^{-1}$. It has survived four months in a 4 °C cold room under 3 μmol photons $\cdot m^{-2} \cdot s^{-1}$ and is tolerant to cryopreservation (50 days at –80 °C with and without added glycerol). In preliminary evaluations of different growth media, sodium thiosulfate, 1.0 μM $Na_2S_2O_3 \cdot 5H_2O$, was added to culture media because the atmosphere above some ponds at the SPNWR smelled of reduced sulfur compounds and thiosulfate is a reduced sulfur compound. It was found that thiosulfate was not detrimental to culturing 980625-4A at in both SPNWR natural brine and AS100, an artificial sea water medium. The alga's tolerance to assorted environmental extremes makes it a suitable organism for studying acclimation physiology.

Relatively few scientific investigations have been conducted at the SPNWR. These consist of a plant census (Baalman 1965), a stream habitat study (Meehan et al. 1996) and ongoing research on migrating waterfowl by members of the Department of Zoology at Oklahoma State University. Microbiological studies at the salt plains are being conducted by Professor Ralph S. Tanner of the Department of Botany and Microbiology at the University of Oklahoma. He has isolated and described a new bacterium, *Vibrio aspartigenicus* (personal communication) from soil at the SPNWR. He is conducting biochemical and genetic analyses on *V. aspartigenicus* and other bacterial isolates from the SPNWR. There clearly is a need to investigate microbial communities at the SPNWR salt flats because these communities have not been well documented. New extremophiles await discovery at the SPNWR and there is a growing push to commercially exploit hypersaline organisms (Aguilar et al. 1998).

C. Extremophiles

There is great public and private interest in commercial exploitation of organisms from extreme environments or their products. Extremophiles or substances derived from them are capable of functioning in environments that prevent the growth of many organisms and functions of most enzymes. Enzymes from extremophiles including proteases, amylases, cellulases, lipases, DNA polymerases, and dehydrogenases have found commercial uses, as have antibiotics and plasmids (Pennisi 1997). The biotechnology revolution has depended upon DNA polymerase enzymes isolated from organisms living in hot springs for the polymerase chain reaction (Ventosa and Nieto 1995). Extremophiles and their products are used in a variety of ways to increase the

human quality of life, including uses in saline wastewater treatment plants, as detergent additives, extremozymes for food and chemical processing, production of food for livestock and human nutritional supplements, environmental bioremediation, and coal desulphurization (Ventosa and Nieto 1995, Pennisi 1997, Kargi and Dinçer 1998, Persidis 1998, Hough and Danson 1999). Molecular biologists will benefit from alkaline phosphatases from extremophiles for non-radioactive enzyme assays. Liquid fuel (oil) from halophilic species of the genus *Dunaliella* is also being investigated (Ginzburg 1993). Extremophiles will improve human life in the future as sources of cheaper food and energy, improved detergents, new antibiotics, and light, long-lasting, breathable synthetic fabrics. Such great economic potential promises to drive further discoveries of extremophiles. Most extremophilic microbes studied have been prokaryotes. As a result, there is a great need to discover and characterize more eukaryotic extremophiles for their potential benefits for new technologies. Unicellular algae are some of the most promising eukaryotes to be investigated for these purposes because they are easily cultured in the laboratory, acclimate to environmental stresses, and the physiology of *Dunalliella* has been extensively studied providing a baseline for future investigations.

Most life forms have evolved to survive in a narrow range of environmental conditions. Hot springs, hydrothermal vents and polar ice are extreme environments, but the temperatures and other conditions encountered by organisms in these environments are relatively constant through time. The most remarkable organisms are those that can adapt to rapid and dramatic changes in environmental conditions. A new term, poikilotroph, has recently been defined to describe these extremophilic organisms (Gorbushina and Krumbein 1999). Poikilotrophs are the "super" organisms of the world.

They have not only adapted to combinations of harsh conditions (extremes of salinity, temperature, dissolved oxygen, pH, photon flux density, and possibly nutrient limitation) but must continually acclimate to dramatic changes in these environmental stresses. Some of the most exciting poikilotrophs are organisms which inhabit hypersaline waters because these environments exhibit frequent and rapid transitions between high and low temperatures, dissolved oxygen and nutrient levels, light intensity, osmolarity, and pH. Under such circumstances, halotolerance alone is probably insufficient for survival, rather halophiles and halotolerant organisms must adjust physiological functions to environmental fluctuations.

The defining characteristic of halophiles is maintenance of osmotic balance between the protoplast and the saline environment. There are several ways that such organisms maintain osmotic balance during increases in extracellular salinity: uptake of ions (mainly by Archaea), extrusion of ions, production of molecular osmolytes, and combinations of these methods (Ben-Amotz 1975, Galinski et al. 1997, Gilles 1997, Silva et al. 1999).

D. Algae as Extremophiles

Dunaliella is the most intensively studied halophilic algal genus. It's species are able to live in nearly the entire range of salinities found in hypersaline environments (approximately 0.34-5.98 M NaCl), and in pH 0 (*D. acidophila*), at –6 °C (*D. antarctica*), and at high irradiances (*D. bardawil* Ben-Amotz et Avron) (Pick 1998, Gokhman et al. 1999). *Dunaliella* is probably the most abundant eukaryotic microorganism in many hypersaline environments because it lacks a rigid cell wall, enabling it to withstand

sudden hypo- or hyperosmotic changes up to three-fold (Pick 1998). It has been touted as "a model extremophilic alga" because it adapts to extreme environmental conditions with both short-term and long-term responses (Pick 1998). A short-term response to an increase in salinity is production of glycerol as the compatible/compensatory solute for osmotic balance. Upon hypoosmotic shock *Dunaliella* responds with a rapid release of glycerol into the environment (Fujii and Hellebust 1992, Imhoff 1993). Long-term responses to increases in salinity include increased β-carotene synthesis in *D. bardawil* and size alternations in organelles (Marín 1998, Berube et al. 1999).

E. Taxonomy of the Green Algae

Perhaps the most important aspect of taxonomy is that it promotes formation of hypotheses regarding relatedness of organisms, evolutionary processes, and biological function. Unfortunately, the evolutionary record is incomplete, relatedness is subjective, e. g. the members of the genera *Chlorella* are not closely related and are currently divided into two classes, the Trebouxiophyceae and Chlorophyceae (National Center for Biotechnology Information 2000), and the functions of many genes remain largely unexplored. Modern technologies, including the electron microscope, DNA sequencing, and biochemical analytical tools, are providing new information about relatedness and function that should improve classifications and lead to novel biological hypotheses.

Taxonomy enables a researcher to identify an organism and to recognize when an organism has not yet been described. Characteristics used to classify algae include morphology, ultrastructure, modes of cell division, life cycles, chemical compounds and DNA sequences. In the past, descriptive morphology was heavily relied upon for

classification. More recently a comprehensive approach taking advantage of technological advances is becoming the standard for algal taxonomy (Zahn 1984, Geisert et al. 1987, Albertano et al. 1991, Kessler and Huss 1992, Gladu et al. 1995, Hegewald and An 1998).

The diversity of algae is extraordinary, and the chlorophytes are considered the most diverse of algal groups (Mattox and Stewart 1984), but the number of measurable characteristics in this group is somewhat limited this causes problems in classification because of simple body forms and some algae have life unicellular forms in their life cycles. Bold and Wynne (1985) list 14 schemes for naming and describing algal Divisions and Classes and Vymazal (1995) lists five additional Division and Class schemes. Phycologists have also used different variations of Division names (Bold and Wynne 1985).

Along with various propositions for organizing algal taxa, there are differing ideas regarding which algal features are important in classification. For example, Lee (1989) proposed that the Order Chlorococcales is "exclusively freshwater", van den Hoek et al. (1995) writes of the Order Chlorococcales that "...only a tiny minority are marine...", whereas Bold and Wynne (1985) did not mention salinity as a defining factor for the order. Without consensus among phycologists, taxonomy will continue to be confusing. This is particularly true when one is classifying algae capable of living in a broad range of salinities.

There are several genera of coccoid green algae able to grow in fresh water, marine and elevated saline environments. *Dunaliella*, *Chlorella* Beijernick 1890, and *Chlamydomonas* Ehrenberg 1833, are the most common unicellular, halophilic and or

halotolerant chlorophytes used in stress tolerance studies (Loseva et al. 1998, Loeblich 1982). *Dunaliella* and *Chlorella* include species and or strains found in fresh, brackish, euryhaline, marine and hypersaline environments. Compared to the vast literature describing physiological responses to stress, there are only a few studies (excluding *Dunaliella*, *Chlorella* and *Chlamydomonas*) involving unicellular halophilic or halotolerant unicellular chlorophytes.

Members of the genus *Dunaliella* are unicellular, biflagellate green algae (Chlorophyta), and are composed of 28 described species: five freshwater species, all believed to be rare, and 23 saline species. *Dunaliella* has been extensively studied in the past four decades and most physiological aspects have been and are currently being investigated, e. g. salinity responses (Johnson et al. 1968, Ben-Amotz and Avron 1973, 1979, Golldack et al. 1995, Berube et al. 1999), cellular membranes (Ginzburg 1969, Weiss and Pick 1996, Chitlaru et al. 1997), environmental factors (Van Auken and McNulty 1973, Ben-Amotz 1975), lipids (Tornabene et al. 1980, Lynch and Thompson 1984), photosynthesis (Ben-Amotz and Avron 1972, Hootkins and Bearden 1983, Morissette and Popovic 1987, LaRoche et al. 1991, Durnford and Falkowski 1997, Melis et al. 1999), enzymes (del Rio et al. 1994) and the study of the 1992 bloom in the Dead Sea (Oren et al. 1995).

The non-flagellated, coccoid genus *Chlorella* is composed of approximately 14 described species, mainly soil or freshwater members with few being marine (Kessler 1982). The soil and freshwater members range in diameter from 2-12 μm and the marine species range in diameter from 1-2 μm. They are a genetically diverse group with species-specific biochemical and physiological properties (Kessler 1982, van den Hoek et

al. 1995). *Chlorella* propagate by autosporulation forming two to several autospores from one parent cell. Autosporulation is the formation of two or more daughter cells (autospores), each identical to the mother cell except smaller, and delineated by their own cell wall which is independent of the mother cell wall. *Chlorella* contains a sporopollenin-like substance (polymerized carotenoid) in its cell wall and the chloroplast contains a pyrenoid. Eighty-eight strains of *Chlorella* are able to live in a wide range of salinites from 0-0.85 M NaCl. There are a few species able to grow at 38-42 °C, such as *C. sorokiniana* (Kessler 1982).

Classification of coccoid green algae is needed to establish similarities and differences within the Division Chlorophyta. Current taxonomy in coccoid green algae is uncertain and often confusing. For example, algae of the genus *Chlorella* are among the most abundant coccoid green algae occurring in both fresh and marine waters, but nominal *Chlorella* spp. are now distributed in the Classes Chlorophyceae and the Trebouxiophyceae. *C. saccharophila* is placed in the Trebouxiophyceae along with *C. vulgaris* Beijernick 1890 and *C. sorokiniana*. *C. zofingiensis* is placed in the Chlorophyceae. There are five *Chlorella* spp. and two *Nannochloris* spp. in the Class Trebouxiophyceae that are unassigned to Order and Family (National Center for Biotechnology Information, 2000). This situation illustrates the uncertainty and confusion regarding how to classify these organisms. Clearly *Chlorella* is not a feasible taxon as it presently exists.

Among the common coccoid green algae, the small size of the alga used in this study, designated 980625-4A, is atypical of algae in the genera *Oocystis* Nageli 1855,

Pycnococcus, Trebouxia de Puymaly 1924, but is characteristic of *Nannochloris* and the smaller *Chlorella* species. Characteristics of the former genus are described below.

F. The genus *Nannochloris* Naumann 1919

Members of the genus *Nannochloris* Naumann 1919 are very small among green algae, approximately 1-5 μm in diameter, unicellular, coccoid, non-motile and morphologically indistinct from many small unicellular coccoid green algae. Classification of unicellular coccoid green algae has been an evolving and controversial process, particularly where the genus *Nannochloris* is concerned.

The genus *Nannochloris* and two species, *Nannochloris bacillaris* Naumann and *Nannochloris coccoides* Naumann (Chlorophyta), were described by Naumann (1919). The translation of the name *Nannochloris* is "little grass." Naumann described the genus *Nannochloris* as being unicellular and dividing by binary fission, a type of cell division where the mother cell divides into two identical daughter cells delineated by the mother cell wall. *Chlorella* is a common unicellular green alga with great morphologic similarity to *Nannochloris*. The only aspect Naumann used to distinguish between the two genera was the mode of cell division. *Chlorella* divides by autosporulation, where the mother cell divides into two identical daughter cells (autospores) which form their own distinct cell wall separate from the mother cell wall.

In 1941, Pennington described a new species *Diogenes rotundus* (Fritsch 1949), but Fritsch moved it to the genus *Nannochloris* and renamed it *Nannochloris rotundus* (Pennington) Fritsch comb. nova. Fritsch believed Pennington did not take the size of the alga into consideration when classifying *D. rotundus*. Fritsch reclassified *D. rotundus* to

N. rotundus because it was most likely "identical to *N. coccoides.*" Butcher (1952) described two marine species, *N. maculatus* and *N. atomus*. Droop (1955) described *N. oculata*. Classification of these algae became more complicated when Hibbert (1981) found that *N. oculata* did not contain Chlorophyll *b* (Chl *b*) like the other *Nannochloris* spp. On this basis, he re-assigned *N. oculata* to the Class Eustigmatophyceae and renamed it *Nannochloropsis oculata* (Droop) Hibbert (Sarokin and Carpenter 1982). In 1980, Tschermak-Woess described *Nannochloris normandinae*, the phycobiont of the lichen *Normandina pulchella* (Miyachi et al. 1989). At about the same time, Menzel and Wild (1989) re-classified *Nannochlorum eucaryotum* Wilhelm et al. 1982 as *Nannochloris eucaryotum* (Wilhelm et al.) Menzel and Wild comb. nova based upon comparative ultrastructure, physiology and biochemistry (Menzel and Wild 1989). Krienitz et al. (1996) concluded the alga *Choricystis minor* was a synonym for *Nannochloris coccoides*. Tschermak-Woess (1999) explained that the lectotype of *Nannochloris*, *N. coccoides*, established by Hibbert (1981), is actually *Marvania geminata* Hindak 1976, which multiplies by budding, and proposed *Nannochloris eucaryota* (note spelling) as the replacement. A lectotype is a replacement specimen for the missing or destroyed holotype.

The mode of algal cell division remains an important distinguishing attribute for some scientists. However, electron microscopy has revealed autosporulation among algae assigned as *Nannochloris* spp., whereas binary fission is supposedly a defining characteristic of this genus (Krienitz et al. 1996). Four different research groups, using TEM micrographs, have documented autosporulation in *N. bacillaris, N. atomus, N. maculatus, N. coccoides* and other *Nannochloris* spp. (Sarokin and Carpenter 1982,

Brown and Elfman 1983, Menzel and Wild 1989, Tschermak-Woess 1999). Several scientists have also documented exclusively binary fission in *N. bacillaris* using TEM micrographs (Ogawa et al. 1995, Krienitz et al. 1996). A reevaluation of the genus *Nannochloris* using biochemical taxonomy has been proposed (Sarokin and Carpenter 1982, Brown and Elfman 1983). Obviously there is controversy surrounding the definition of the genus *Nannochloris*. Krienitz et al. (1996) have been vigorous advocates for keeping the genus *Nannochloris* as Naumann originally described it, including only those organisms displaying binary fission. In contrast to this conclusion and the observations of Naumann, Menzel and Wild (1989) state that the genus "*Nannochloris* Naumann is [sic. has the] characteristic of autosporulation."

The genus *Nannochloris* contains diverse algae and among the many isolates assigned to the genus *Nannochloris* only six or seven have been assigned to species. Some species occur in freshwater and others are marine, and one species is a phycobiont (Fritsch 1949, Brown and Elfman 1983, Menzel and Wild 1989, Miyachi et al. 1989). Some species contain a pyrenoid and some do not (Brown and Elfman 1983). It is obvious the genus *Nannochloris* needs further study and revision.

The uncertainty in assignments to the genus *Nannochloris* based on mode of cell division and morphology highlight the need for more extensive characterization. In particular, classifications of these algae ought to be based more upon DNA sequence analysis and biochemical features such as enzyme activities, pigment composition, and, in the case of halotolerant algae, osmolyte identity.

The objectives of this thesis research were to identify the unicellular coccoid green alga collected from the SPNWR with electron microscopy, pigment and osmolyte analysis, and to elucidate relationships between growth rates, salinity and temperature.

G. Salinity and Temperature Stress in Algae

Physiological adaptations to environmental stress(es) may divert the cell's energy away from reproduction processes and reduce its growth rate. Complex mechanisms regulate response(s) to stress, determining compensation and tolerance to poikilo-environments. The concurrent interactions of several mechanisms make investigations into stress physiology complex. Studying a limited number of physiological responses to salinity and temperature stress will not uncover all the secrets of the poikilotroph but may reveal some important adaptative mechanisms.

Elevated solute concentrations are not inherently stressful to halophilic or halotolerant organisms, but changes in environmental osmotic conditions can cause osmotic stress. Hypersaline shock leads to sodium and chloride toxicity and initiates stress adaptation processes and their regulatory pathways (Imhoff 1993, Serrano et al. 1996). These processes include active transport out of the cell through plasmalemma proteins, production of compatible/compensatory solute(s) (osmolytes), and synthesis of stress-defensive proteins (Serrano et al. 1996). Osmolyte synthesis involves manufacture of solute(s) to maintain osmotic equilibrium and protect cellular components (Galinski et al. 1997). The term "compatible" refers to its relationship to protein function (Brown 1976). Osmolytes are called compensatory solutes because they neutralize the harmful effects of saline stress (Clark 1985).

Substantial energy costs are involved in both active transport of ions and osmolyte synthesis (Brown 1982a). Both are expected to be elevated under hypersaline conditions. In most halotolerant and halophilic organisms, moderate changes in salinity may have a high energetic cost, resulting in a generally reduced maximum growth rate (Tindall et al. 1978, Gorbushina and Krumbein 1999). Algal specific growth rates (μ, d^{-1}) have been used to evaluate the impact of environmental stress(es) (Talling 1955, Tomas 1978, Senft et al. 1981, Fawley 1984, Ostroff et al. 1980). Growth rates of the marine diatom *Cyclotella cryptica* (Liu and Hellebust 1976) and the marine *Nannochloris bacillaris* (Brown 1982a) decreased with increased salinities.

Temperature stress initiates the synthesis of heat shock proteins (HSP) and increases the synthesis of constitutive HSP in plants and animals (Brodl et al. 1994). Cellular lipid composition, protein-lipid interactions, enzyme activities, and rates of other biochemical processes are influenced by temperature (Mohanty et al. 1985, Raven and Geider 1988). Temperature changes affect many physiological processes and can exert tremendous stress on an organism.

Algal responses to temperature have been investigated for over half a century. The model halophilic alga, *Dunaliella,* increased its doubling time from 23.8 h at 32 °C to 41.5 h at 40 °C (Van Auken and McNulty 1973). The flagellated unicellular green alga *Chlamydomonas reinhardtii* produces a set of heat shock proteins (HSP) when temperature or salt stressed. Both stresses induce a specific set of HSP and both elicit the synthesis of a 70 kDa protein (Adams 1991). Antarctic cyanobacteria displayed a reduced carotenoid/Chlorophyll *a* ratio with increased temperatures (Roos and Vincent 1998). Carotenoids are thought to play a protective role in the thylakoid membranes

(Havaux 1998). Growth rate of the cryptomonad *Cryptomonas ovata* Ehrenberg 1838, increased at temperatures up to an optimum temperature, then declined at higher temperatures (Cloern 1977).

There is much to be learned from studying microalgae capable of living in extreme environments, including the rarely studied poikilotrophic green algae, which live and sometimes thrive in widely fluctuating environmental conditions. Investigations into the adaptations to environmental extremes will, in time, provide explanations to physiological processes under moderate conditions (Osmond 1987) and may lead to agricultural advances. Extremophiles may provide molecular biology with novel molecules to exploit and industries with enzymes to improve human life.

H. Summary of Thesis Objectives

My work aimed to isolate and identify a halotolerant unicellular coccoid green alga from the Salt Plains National Wildlife Refuge, Alfalfa County, Oklahoma. An additional objective was to characterize the responses of this alga to elevated salinities and temperature. The following section describes the isolation and identification of the alga, pigment and osmolyte analyses and describes growth rate, cell size, and ultrastructure changes in the green alga in response to salinity and temperature stresses.

MATERIALS AND METHODS

A. General Laboratory Procedures

All chemicals used were of reagent grade and were purchased from Aldrich, Cole-Parmer, Fisher Scientific, Pharmco, or Sigma Chemical companies.

Glassware was washed prior to initial use in concentrated sulfuric acid to remove resistant organic contaminants such as greases, followed by scrubbing with warm water and detergent, five rinses with tap water, three rinses with Nanopure water to remove additional residues. The Erlenmeyer flasks were capped with a triple layer gauze-wrapped cotton plug and covered with Al foil to exclude airborne contaminants. The flasks were autoclaved at 16 to 18 psi, 125 °C for 25-30 minutes, which is standard procedure to ensure sterilization. The time was increased when more than 2 L of medium was autoclaved at one time. All Erlenmeyer flasks used for culturing were 125 mL unless otherwise stated.

Nanopure water greater than 17 MΩ-cm was used for all media preparation to minimize contamination with ions and organics.

All polycarbonate, polyethylene or polypropylene containers were soaked for 24 h in 1 M HCl for 24 h to remove minerals and some organics from the surfaces followed by thorough rinsing with Nanopure water.

The photon flux density (PFD) of the laboratory cool white fluorescent lamps used for culture maintenance and experiments was ~50 μmol photons $\cdot m^{-2} \cdot s^{-1}$ on a 14:10 Light:Dark (L:D) cycle, (approximately summer photoperiod in Oklahoma) unless stated otherwise.

B. Collection of algae from the SPNWR

Planktonic algal samples were collected at different locations on the SPNWR throughout the year in order to increase algal diversity for cataloging and possible experimental organisms. Algal samples were collected in polyethylene screw capped bottles, labeled and transported (~2.5 h) from the SPNWR to the laboratory in the dark at ambient automobile temperatures. In the laboratory, the algal samples were given an identification number for documentation. All field data (e. g. collection sites) and algal identification numbers were recorded in a laboratory notebook for future reference. The identification number is composed of:

YYMMDD-site number-letter of sub-sample

The sub-samples were produced either by dividing the sample in the laboratory or from multiple samples collected at one site. For example, 980625-4A and 980625-4B: the sample was collected on June 25, 1998 from site 4 (Fig. 2). In an effort to produce axenic cultures from the algal sample, two different colonies, "a" and "b," were removed from an agar Petri dish and transferred into a liquid medium and designated 980625-4Aa and 980625-4Ab.

The planktonic algal samples in the original collection bottles were placed with unscrewed caps on shelves under cool white fluorescent lamps.

C. Sources of algae used in this study

The unicellular coccoid green alga (Chlorophyta) used in this investigation was collected on June 25, 1998 from the SPNWR by Dr. William J. Henley, Department of Botany, Oklahoma State University, and was designated 980625-4A.

Chlorella saccharophila UTEX 2469, *Chlorella sorokiniana* UTEX 1810, and *Chlorella vulgaris* UTEX 1809 were obtained from The University of Texas, Austin Algal Collection.

D. Maintenance of algal cultures in the laboratory

Algal cultures were maintained in the laboratory in Salt Plains (SP) medium prepared from salts collected at the SPNWR at 50 g-L^{-1} total dissolved solids, in liquid medium as well as agar plates and slants. The exact concentration of NaCl in SP is uncertain, therefore concentrations are expressed using g-L^{-1} total dissolved solids. Salinities of the defined medium AS100 are expressed in g-L^{-1} NaCl to facilitate comparisons between SP and AS100. The salinity of 50 g-L^{-1} total dissolved solids was chosen because it allowed lush growth of an assortment of SPNWR algae already in culture, and is a moderate salinity relative to Salt Plains pools. When liquid cultures were rich in algal cells, an aliquot was transferred to fresh SP medium.

Complete chemical characterization of SP salts has not yet been performed, but trace elements were analyzed by Centre Analytical Laboratories (State College, PA) using inductively coupled plasma-mass spectrometry (ICP-MS; Table 1). Water samples from the SPNWR were diluted 1:200 in Nanopure water before analysis to reduce the total salt concentration to within the working range of the ICP-MS instrument. The salt crystals were dissolved in Nanopure water at a concentration of 0.484 g salt in 100 mL Nanopure water.

Table 1. Trace elements in brine and salts collected at the Salt Plains National Wildlife Refuge, Oklahoma, USA. Analyses were performed by ICP-MS.

Element	SPNWR Water (30 g-L^{-1} total dissolved solids)	Salt Crystals	Nanopure Water Blank
	µg/L	µg/kg	µg/L
Al	LOD	401	<LOD
As	250	11384	89.8
B	<LOD	2397	0
Ba	2120	1236	346
Br	1.01E+05	3.39E+05	<LOD
Cd	<LOD	727	<LOD
Ce	<LOD	0	<LOD
Cl	2.90E+08	1.10E+10	<LOD
Co	<LOD	135	<LOD
Cr	143.6	820	138
Cu	145.4	748	<LOD
Fe	3960	153926	<LOD
Hg	27.2	<LOD	<LOD
K	3.62E+05	1.66E+06	<LOD
La	<LOD	<LOD	<LOD
Li	1064	4050	<LOD
Mg	1.11E+06	3.43E+06	604
Mn	576	71694	332
Mo	<LOD	<LOD	<LOD
Na	out of range	out of range	3.22E+05
Ni	<LOD	607	<LOD
P	<LOD	<LOD	<LOD
Pb	1250	940	<LOD
Rb	<LOD	285	<LOD
S	1.64E+06	1.08E+08	<LOD
Se	146.8	1277	<LOD
Sr	3.74E+04	2.81E+05	<LOD
Tl	102.6	34	<LOD
V	410	10310	272
Zn	400	3182	0

*<LOD = below method limits of detection

Concentrated SP brine was prepared by dissolving salt nodules collected from salt saturated ponds or salt crystals collected from the sand surface in Nanopure water. After the salts were dissolved, the liquid was filtered under vacuum through a GF/F filter to

remove soil particles, plant and animal debris, and algae. The salinity of the filtrate was measured with a hand held salinity refractometer, after dilution with Nanopure water in the case of overscaled salinities (> 100 g-L^{-1} total dissolved solids). The filtrate was stored in polyethylene bottles and refrigerated to reduce organismal growth.

SP medium was prepared by diluting concentrated SP brine with Nanopure water to the desired salinity. The medium was autoclaved then allowed to cool at room temperature. Inside the laminar flow hood, using sterile technique, autoclaved f/2 nitrate and phosphate stock solutions and filter sterilized f/2 vitamins were added to the cooled SP medium. The medium was swirled to evenly distribute the nutrients then aliquoted into sterilized flasks. Unused portions of SP media were stored at room temperature.

In preliminary evaluations of media composition needed to sustain growth of 980625-4A, sodium thiosulfate, $Na_2S_2O_3 \cdot 5H_2O$, was added at 1.0 μM to both SP medium and AS100, an artificial sea water medium. The atmosphere above some ponds at the SPNWR smelled of reduced sulfur compounds such as hydrogen sulfide, suggesting sulfur reduction occurred in sediments. Thiosulfate is a reduced sulfur compound that can serve as a free radical scavenger, and may protect cells from toxic free radicals. This substance was only used during isolation of the alga, and was not added to media used for later physiological experiments.

Agar (Bacto-Agar, Difco Laboratories, Detroit, Michigan) was washed according to Waterbury and Willey (1988) to remove possible toxic substances, e. g. phenols, humic and fulvic acids which can inhibit growth of organisms. One hundred g of agar was washed 3-4 times in a 4 L glass Erlenmeyer flask (20 minutes on a stirrer) with 3 L Nanopure water until effluent was clear. After the final washing with Nanopure water,

the solution was vacuum filtered through a Buchner funnel with a paper filter to remove as much of the water as possible from the rehydrated agar. The agar was then washed once for 20 minutes with 3 L of 95 % ethanol and filtered in the same manner as previously, followed by one washing for 20 minutes with 3 L of acetone to remove polar and intermediate polarity organics. The washed agar was dried at 50 °C overnight in a glass baking dish and, to prevent condensation, cooled to room temperature inside the oven. The agar was stored in a polyethylene screw cap bottle at room temperature.

Agar plates were prepared in the following steps: 15 g of washed agar (Waterbury and Willey 1988) was added to 1 L of SP medium (minus f/2 nutrients, Guillard and Ryther 1962) in a 2 L glass or polypropylene Erlenmeyer flask. The flask was covered with Al foil, autoclaved and removed to a 55 °C water bath. After approximately 45 minutes, the flask was removed from the water bath and transported to the laminar flow hood. f/2 nutrients (Guillard and Ryther 1962) were added and the flask was gently swirled to thoroughly mix the added nutrients. The warm liquid agar was poured into ~40 sterile plastic Petri dishes (100 x 150 mm). The agar in the Petri dishes was allowed to cool and solidify in the laminar flow hood for ~ 30 minutes. The lids of the Petri dishes covered half of the bottom Petri dishes to prevent excess water accumulation on the inside of the Petri dish covers. The cooled solidified agar plates were covered with their lids and placed into the original Petri dish plastic bag, sealed with laboratory tape, labeled and either used directly or placed into a 4 °C cold room to prevent desiccation. As needed, agar Petri dishes were allowed to equilibrate to room temperature (~30 minutes) to prevent temperature shock to the organisms.

Agar slants were maintained in twenty-five mL screw cap glass test tubes. The test tubes were filled approximately half full with SP, 50 g-L^{-1} total dissolved solids agar medium. The tubes were cooled uncapped in a test tube rack lying on its side with one edge elevated approximately 1.5 cm allowing the liquid agar to form the largest surface area without touching the inside top of the test tube. After cooling to room temperature, the tubes were capped and sealed inside labeled plastic bags and placed in a 4 °C cold room to prevent desiccation. As needed, agar slants were equilibrated to room temperature (~30 minutes) to prevent a temperature shock to the organisms.

After a considerable amount of agar was covered with algal cells, approximately six weeks, the agar plates and slants were sealed with Parafilm, dated and archived into the 4 °C cold room under continuous ~3 µmol photons $\cdot m^{-2} \cdot s^{-1}$.

E. Isolation of algae into clonal axenic cultures

A clonal axenic culture starts with one cell and contains a single genotype of the organism. Standard microbiological procedures are successful, time tested and were followed to produce clonal axenic cultures (Yopp et al. 1978). All algal transfers were performed inside a laminar flow hood prepared in the following manner: the hood was turned on and the inside surfaces were sterilized with 70 % ethanol and the hood remained on for 15 minutes before use. Cooled, flame-sterilized implements were used when working with algal cultures.

Liquid algal cultures were streaked onto solid agar plates for isolation. The outer edge of the bottom dish was labeled with an indelible ink pen. The goal of streaking a plate for isolation is to reduce the number of organisms with each successive streak. In

the final streaked area, individual cells should be separate from other cells. These isolated cells should divide and develop into clonal colonies to be transferred to another sterile medium to continue the growth of the clonal colony. A total of three or four consecutive streaks were made per agar plate, flame sterilizing the metal loop after each streak. The streaked isolation plates were incubated at 20-25 °C under cool white fluorescent lamps.

When isolated colonies developed to a size of approximately one mm diameter on the agar plate, the colonies were removed from the agar surface with a sterilized wooden toothpick and placed in separate sterilized 15 mL conical centrifuge tubes with approximately 5 mL of SP, 50 g-L^{-1} total dissolved solids liquid medium. The tubes were capped with a double gauze wrapped cotton plug and placed in a test tube rack under cool white fluorescent lamps.

After incubation, the culture was examined under a microscope to determine what microorganisms were present. If several different organisms were observed, the liquid culture was then used to streak more agar plates for isolation. The isolation procedure continued until axenic clonal cultures were produced, confirmed by microscopic inspection.

Antibiotics were also used to produce axenic cultures. One hundred mL of an antibiotic cocktail was prepared from the following antibiotics: 2.5 g penicillin G, 2.5 g ampicillin, 0.25 g carbenicillin, 0.25 g d-cycloserine, and 63 mg vancomycin (Brown 1982b). Four mL of the cocktail were added to each of three mid-logarithmic cultures, 50 mL of SP 50 g-L^{-1} total dissolved solids. Two days later, 1 mL of each culture was aliquoted into individual microcentrifuge tubes and centrifuged at 5000 x *g* for three

minutes to separate the cells from the antibiotic medium. The supernatant was removed from the microcentrifuge tubes and discarded. One mL of AS100, 0 g-L^{-1} NaCl was added to the microcentrifuge tube and the cells were vortexed vigorously for five to ten seconds. The algal cell walls are not affected by the antibiotics and are able to withstand the hypoosmotic shock and vortexing. AS100 is a defined artificial salt water medium (University of Texas, Austin, Algal Collection) and was used as the transfer medium because AS100 can be prepared without adding NaCl, whereas SP medium, made from dissolved salt crystals from the SPNWR, cannot. The bacterial cell walls are compromised by the antibiotics and cannot withstand these stresses. The cells in AS100 0 g-L^{-1} NaCl were transferred into separate flasks containing 50 mL of SP, 50 g-L^{-1} total dissolved solids. Axenic cultures were examined ten days later under a light microscope to determine the level of bacterial contamination.

F. Experimental culture medium, AS100

The culture medium for most experiments was the defined artificial sea water medium, AS100, which was altered for the culturing of L1644 *Dunaliella* by the University Texas, Austin Algal Collection. f/2 vitamins were added aseptically to the cooled medium (Guillard and Ryther 1962).

G. Microscopy of 980625-4A

The surfaces of 980625-4A algal cells and cell size were documented with scanning electron microscopy (SEM) and the ultrastructure was examined by

transmission electron microscopy (TEM). The SEM and TEM protocols were performed at the OSU Electron Microscopy Laboratories.

980625-4A cells were harvested from cultures in mid- to late-logarithmic growth phase grown in AS100, 0 g-L^{-1} NaCl, and submitted for SEM analysis. Algal cells were collected by centrifugation. The supernatant was discarded and the cells were resuspended in 2 % phosphate buffered glutaraldehyde. After two hours, the suspension was gently centrifuged and the supernatant was discarded. Cells were washed and centrifuged twice in fresh 0.1 M phosphate buffer. The cells in the last 0.1 M phosphate buffer were stored in the dark at 25 °C. Glass microscope slide cover slips were cleaned with methanol followed by water, coated with polylysine glue and set aside for one minute. The coverslips were washed with water again and air dried for one minute. The fixed cells were placed on the prepared cover slip and set for ten minutes on the laboratory benchtop at 25 °C. The cover slip with adhered cells was washed three times for five minutes each in 0.1 M phosphate buffers then dehydrated through washing with an ethanol series (50, 70, 90, 95, and 100 %) three times, for ten minutes each. The cover slips with cells were critical point dried using CO_2 to remove all traces of water, then mounted on an aluminum stub and sputter coated with a gold-lead coating.

The following cultures of 980625-4A were acclimated to the specified medium, salinity and temperature before harvesting in mid- to late-logarithmic growth phase for TEM analysis:

A) AS100, 0 g-L^{-1} NaCl, 25 °C

B) AS100, 40 g-L^{-1} NaCl, 25 °C

C) AS100, 80 g-L^{-1} NaCl, 25 °C

D) AS100, 120 g-L^{-1} NaCl, 25 °C

E) AS100, 0 g-L^{-1} NaCl, 40 °C

F) AS100, 40 g-L^{-1} NaCl, 40 °C

G) AS100, 80 g-L^{-1} NaCl, 40 °C

H) AS100, 120 g-L^{-1} NaCl, 40 °C

I) SP, 120 g-L^{-1} total dissolved solids, 25 °C

J) SP, 50 g-L^{-1} total dissolved solids, 25 °C

Algal cells for TEM analysis were gently centrifuged to concentrate the cells. The supernatant was removed and discarded and 2 % phosphate buffered glutaraldehyde was added to resuspend and fix the cells. The cells were fixed for two hours at 25 °C then centrifuged. The supernatant was removed and the cells were washed twice in 0.1 M phosphate buffer. The pellet was post-fixed in 1 % aqueous osmium tetraoxide for two hours at room temperature then centrifuged. The supernatant was removed and the pellet was washed twice in 0.1 M phosphate buffer. The cells were gently centrifuged and dehydrated through a graded acetone series (50, 70, 90, 95, and 100 %) three times, for twenty minutes each. After dehydration, the cells were centrifuged, removed from the third 100 % acetone wash and embedded in 1:1 acetone/polybed, capped and left overnight in a ventilation hood. Then fresh polybed was added to the 1:1 acetone/polybed and placed into a 60 °C oven for two days. Thick and thin sections were prepared for TEM analyses.

H. Cell size determination of cells grown at different salinities and temperatures

The size of 980625-4A cells were determined by photographing the cells using a Nikon light microscope, model EFD-3, at 500 x magnification with Kodak ASA 100 color print film. Negatives were developed as 4" x 5" prints which were scanned using a Microtek scanner with 16-bit gray-scale digitization at 600 dots-per-inch. Imaged cell sizes were quantified by digital image analysis on a Power Macintosh G3 computer using the public domain NIH Image software version 1.62 (developed at the U. S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). The major and minor diameters were measured and ratio of major:minor diameters were calculated.

The cells were grown at 25 and 40 °C in AS100 at concentrations of 0, 40, 80, 120 $g\text{-}L^{-1}$ NaCl. A hemocytometer was used as a guide when taking pictures of the cells to ensure a different field of view. Three pictures at three different exposures were taken of each field of view, normal exposure and one *f*-stop below and above the normal exposure to increase the chances of producing a picture with adequate contrast. At least 10 different field of views were documented for each treatment to include at least 150 cells. The picture with the best contrast of the set of three was scanned. The treatment of 25 °C, AS100, 40 $g\text{-}L^{-1}$ NaCl had the largest sample size, n=370, and the smallest sample size was 25 °C 0 $g\text{-}L^{-1}$ NaCl, n=109. There are pseudoreplicates because each treatment consisted of a single flask.

Statistical treatment (2-Way ANOVA and Kolmogorov-Smirnov One Sample Test using Normal distribution) of cell size measurements was performed using SYSTAT software, version 9.01 (SPSS, Inc. 1999).

I. Regression of A_{750} vs. cell counts

Daily microscope cell counts were performed on 13 cultures grown in SP, 50 $g\text{-}L^{-1}$ total dissolved solids with f/2 nutrients (Guillard and Ryther 1962) using a Levy Chamber Hemocytometer (Henley and Yin 1998) to produce a robust regression of A_{750} vs. microscopic cell counts (n=134) (Fig. 4). Two sets of 80 squares were counted for each culture, then an average was taken and used for the "number of cells" in the following formula:

$$\frac{\text{Number of cells x dilution x 4000}}{\text{80 squares counted}} = \text{cells} \cdot mL^{-2} \times 1000 = \text{number of cells per mL}$$

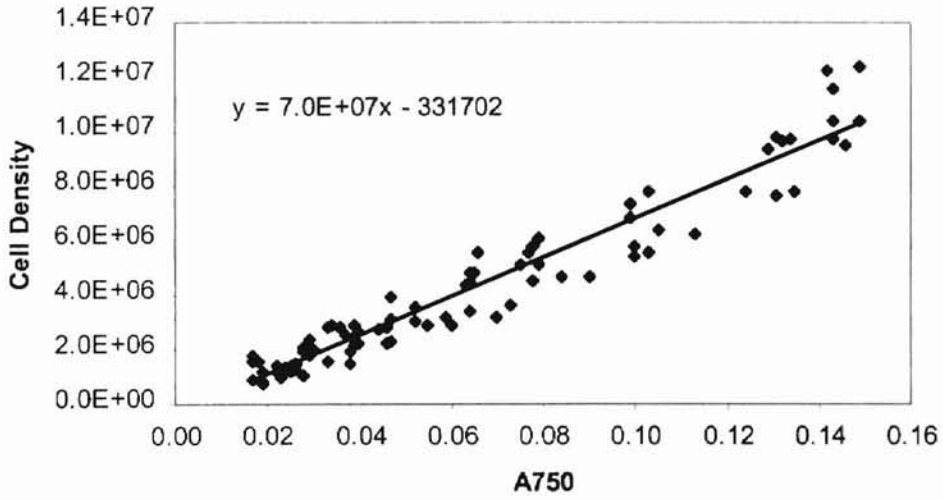

Figure 4. Standard curve of the regression of A_{750} vs. microscopic cell counts of 980625-4A grown in SP at 40 $g\text{-}L^{-1}$ total dissolved solids; n = 88, $r^2 = 0.93$.

The specific growth rates (μ, d^{-1}) were calculated as the slope of the regression of ln(cell density) versus time through the logarithmic growth period, days 2 to 6 for all treatments. The r^2 values for the best fit to the growth for all treatments were greater or equal to 0.9 except for the cultures grown at 25 °C in AS100 at 80 and 120 $g\text{-}L^{-1}$ NaCl, for which r^2 ranged from 0.65 to 0.87 and 0.14 to 0.75 respectively. Cultures grown at 40 °C in AS100 at 0 $g\text{-}L^{-1}$ NaCl gave r^2 values ranging from 0.01 to 0.26.

After the daily cell counts were completed, the absorbance at 750 nm (a measure of scattering without interference from pigments) was recorded using a Shimadzu UV-160U spectrophotometer, using a 1 cm quartz cuvette.

J. Specific growth rates at different salinities and temperatures

980625-4A cells were allowed to acclimate to AS100 before experiments were conducted. Aliquots of mid logarithmic growth phase SP grown cells at 0, 40, 80, 120 $g\text{-}L^{-1}$ total dissolved solids ranged from 3-5 mL and were transferred into 150 mL of AS100 at corresponding salinities, n = 3, to acclimate 980625-4A to the AS100 media. After the AS100 cultures reached mid-logarithmic growth phase, about 5 days, the three cultures were combined into a sterile 500 mL flask, aseptically in the laminar flow hood to ensure the aliquots were of the same cell density. The aliquots were transferred into experimental cultures (n = 4) containing 150 mL of fresh AS100, 0, 40, 80, 120 $g\text{-}L^{-1}$ NaCl. The volume of each aliquot varied to achieve approximately the same initial cell density in all cultures. The 25 °C experimental cultures were placed inside a 60 x 46 cm plastic tub (minus water to eliminate the "edge effect") where 16 grid positions were designated. Initially and daily thereafter, the flasks were placed randomly into the plastic

tub. The daily positions were recorded for future reference. The tub was placed under cool white fluorescent lamps at 100 μmol photons $\cdot m^{-2} \cdot s^{-1}$. The same procedure and identical tub was used for the 40 °C experimental culture flasks. Heat exchange occurred through a closed system of Tygon tubing, wrapped inside along the edge of the tub three times, and a circulating water heater. The water level inside the tub was higher than the meniscus in the flasks and was adjusted daily. The circulating water heater was on a 14/10 Day:Night cycle, identical to the fluorescent lamps, thus the 40 °C treatment was at 25 °C at night. The 25 and 40 °C tubs were placed side by side, on the same laboratory bench, under the same bank of fluorescent lamps. Each culture flask was swirled at least twice daily to resuspend the cells. The cell density of all cultures was measured spectrophotometrically at 750 nm on a daily basis at the same time of day, plus or minus one hour. Statistical treatment (2-Way ANOVA) of specific growth rates was performed using SYSTAT software, version 9.01 (SPSS, Inc. 1999).

K. Pigment analysis

A total of six algal species and strains were analyzed for their pigment composition and content: 980625-4A, *Nannochloris* sp. UTEX 2378, *Nannochloris* sp. UTEX 2379, *Chlorella sorokiniana* UTEX 1810, *C. saccharophila* UTEX 2469, *C. vulgaris* UTEX 1809. Two mL of each culture in mid- to late logarithmic growth phase were centrifuged at 2500 x *g* for 10 minutes at 24 °C. The pelleted cells were resuspended in 1 mL of *N,N*-dimethylformamide (DMF), and extracted in darkness at 4 °C overnight. The microcentrifuge tubes were then removed from the refrigerator and placed inside a Savant Speed-Vac to evaporate the DMF under vacuum in near darkness.

The dried extract was frozen at –35 °C for analysis via high pressure liquid chromatography (HPLC) at a later date.

A three solvent elution system was used to separate the pigments. Solvent A was 80 % methanol and 20 % Nanopure water containing 0.5 M aqueous ammonium acetate. Solvent B was 90 % acetonitrile and 10 % Nanopure water (V:V). Solvent C was ethyl acetate. The Hewlett Packard (Palo Alto, CA) model 1100 HPLC system was equipped with autosampler, photodiode array detector, micro flow cell (volume = 600 nL) for microbore HPLC separations, and a BetaSil-C_{18} column (1.0 mm inner diameter, 150 mm in length). The flow through the column was 0.1 mL $\cdot min^{-1}$. The Chlorophyll *a* (Chl *a*; lot number 68H7820) and xanthophyll (lot number 67H4025) standards were purchased from Sigma. The β-carotene standard (lot number HGO9331EG) was purchased from Aldrich. The final concentrations were 1μg $\cdot mL^{-1}$ of each of the pigments in Solvent A. Fifty μL of the pigment standard solution was injected into the HPLC for analysis delivering 50 ng of each pigment per injection.

The solvent gradient followed the SCOR recommendations for analysis of photoplankton pigments (Wright and Jeffery 1997). Slight modifications were made for optimum separations with this specific HPLC column. The solvent gradient was: at time zero 100 % Solvent A and a linear gradient for four minutes to 100 % Solvent B, then a linear gradient to 30 % Solvent B and 70 % Solvent C for 18 minutes followed by a linear gradient to 15 % Solvent B and 85 % Solvent C for 25 minutes. The mobile phase was held at 15 % Solvent B and 85 % Solvent C for 35 minutes followed by a linear gradient to 100 % Solvent B for 40 minutes and followed by a linear gradient to 100 % Solvent A for 45 minutes.

The flow rate was approximately 0.100 mL-min^{-1} through the column, delivered using a pre-injection split with the pump delivering a total flow rate of 1.00 mL-min^{-1}. The pre-injection split was used to ensure that changes in solvent composition during the gradient reached the column quickly. The volume of the solvent mixer is large, about 1 mL, compared to the flow rate through the column.

Photodiode array spectra were collected over the range of 350-950 nm. Carotenoid identification was based on comparisons of spectra and relative retention times for reference standards (Wright and Jeffrey 1997). Identities of lutein and β-carotene were confirmed experimentally based upon matches of HPLC retention times and UV visible spectra with authentic standards.

L. Identification of osmolytes

Two cultures of 980625-4A grown in AS100 at 0 and 40 g-L^{-1} NaCl in late logarithmic growth phase were harvested and frozen for future osmolyte analysis. Cell densities of the two cultures were determined to be within 10% of each other based upon measurements of A_{750} on a spectrophotometer. Aliquots of equal volume were analyzed, allowing direct comparisons of osmolyte levels between cultures. Culture aliquots were thawed and dried in the Speed-Vac. The cells were ground under liquid N_2 using a small mortar and pestle, and the residue was transferred into a 1.5 mL microcentrifuge tube. One mL of 5 % perchloric acid was added to each tube, and tubes were then sonicated for 5-10 seconds and extracted at 4 °C for one hour. The tubes were centrifuged (5 minute at 5000 x *g*), the supernatant was removed, brought to pH 6 with 5 M aqueous potassium carbonate and refrigerated (Hausler 2000). The solution was centrifuged at 5000 x *g* for

5 minutes at 24 °C to separate the potassium perchlorate precipitate. Half of the supernatant was removed and evaporated to dryness using the Speed-Vac and archived at –35 °C. The other half was analyzed promptly using liquid chromatography mass spectrometry.

The Hewlett Packard (Palo Alto, CA; model 1100 HPLC), was equipped with autosampler and an Alltech Allsphere column packed with 5 μm strong cation exchange (SCX) particles (4.6 mm inner diameter by 250 mm in length). Solvent A was 0.15 % formic acid in Nanopure water, pH 2.5, and Solvent B was 88 mM ammonium acetate in 85 %/15 % methanol/Nanopure water, pH 7.3. The total flow rate was 0.7 mL-min^{-1}. The column temperature was 30 °C. The liquid phase timetable was: immediately after the injection of the sample and continuing for 3 minutes, 99 % Solvent A and 1 % Solvent B; then a linear gradient for 12 minutes to 10 % Solvent A and 90 % Solvent B; followed by a 15 minute hold at 10 % Solvent A and 90 % Solvent B; another linear gradient over 2 minutes to 99 % Solvent A and 1 % Solvent B; and finally a three-minute hold at 99 % Solvent A and 1 % Solvent B. A Perseptive Biosystems Mariner (Framingham, MA) mass spectrometer was operated using Atmospheric Pressure Chemical Ionization (APCI) in positive ion mode for analysis of osmolytes. The APCI process involves evaporation of the liquid mobile phase after it elutes from the HPLC column using a combination of heat and nebulization with nitrogen gas. Ionization of compounds eluting from the HPLC column is enhanced by use of a corona discharge from a needle held at 5500 volts. Mass spectra were acquired at a rate of 2.0 seconds per spectrum. Osmolyte analysis utilized reconstructed ion chromatograms (RICs) calculated for masses corresponding to common osmolytes plus either H^+ or NH_4^+. Compounds with basic groups such as amino acids

generally were detected as $[M+H]^+$, and compounds such as glycerol lacking basic groups were detected as $[M+NH_4]^+$ ions. The ammonium ion was provided by ammonium acetate in the mobile phase.

Areas under the peaks in the reconstructed ion chromatograms eluting at the same time as the corresponding osmolyte standard were integrated to provide a measure of the amount of the major osmolytes. Peak areas were compared to areas of peaks for a standard solution of several water-soluble compounds. A standard for glucosylglucose was not included and the glucose standard was used instead to calculate the glucosylglycerol concentrations. The osmolyte standard solution was prepared in 10.0 mL of 0.15 % formic acid (pH 2.5) with the following concentrations of compounds ($\mu g \cdot mL^{-1}$): proline 330, glutamate 200, glycerol 330, lysine 110, serine 140, β(D)-glucose 220, and sucrose 520. The concentrations of osmolytes were calculated using normalized A_{750} values to correct for the 10 % difference in absorbance.

M. Classification based upon staining with ruthenium red

Ruthenium red is a dye that stains acidic polymers (pectin) of the cell wall. Ruthenium red has been used as a minor taxonomic marker in classifying *Chlorella* and other small coccoid green algae (Takeda 1991, Puncocharova 1994, Tschermak-Woess 1999).

980625-4A, *Chlorella vulgaris* UTEX 1809, *C. sorokiniana* UTEX 1810, *C. saccharophila* UTEX 1809, *Nannochloris* sp. UTEX 2378 and *Nannochloris* sp. UTEX 2379 cultured in Modified Chu 13 (2x) medium were analyzed for their reaction to ruthenium red.

Two mL of each culture were placed into a microcentrifuge tube and centrifuged at 5000 x *g* for two minutes at 24 °C. The supernatant was discarded and 1 mL of ruthenium red solution, 0.01% ruthenium red in 0.1 % aqueous ammonium chloride (pH 9.0) was added and the cells resuspended (Takeda and Hirokawa 1984). The cells were immediately centrifuged as above and the color of the supernatant was inspected visually. The supernatant was removed, cells were resuspended in 2 mL of sterile AS100, 25 g-L^{-1} NaCl and the color of the medium was determined visually. Then one wash of one mL of sterile AS100, 25 g-L^{-1} NaCl followed by centrifugation was conducted and the color of the supernatant was determined visually.

RESULTS

A. Cell Size

980625-4A is a slightly oval shaped green alga. The cells were measured from scanning electron micrographs, n = 7. The mean major diameter of 980625-4A is 2.1 μm for cells grown at 25 °C in AS100 at 0 g-L^{-1} NaCl (Fig. 5 is in all representative). The major and minor diameters are slightly salinity- and temperature dependent as determined by light microscopy (2-Way ANOVA, $P < 0.05$; Figs. 6-8). Cell size measurements based on light microscopy consistently gave smaller values than those obtained from SEM micrographs. Temperature and salinity-temperature interactions did not statistically affect the ratio of the major:minor diameter (2-Way ANOVA, $P = 0.89$ and 0.08, respectively), although salinity was found to have a statistically significant affect (2-Way ANOVA, $P = 0.00$; Fig. 8). A linear regression of the ratio of the major:minor diameters

of 980625-4A cells grown at 25 and 40 °C and grouped by salinity (pooled temperatures) showed a statistically significant relationship with salinity (slope = –0.00317, $P = 0.001$).

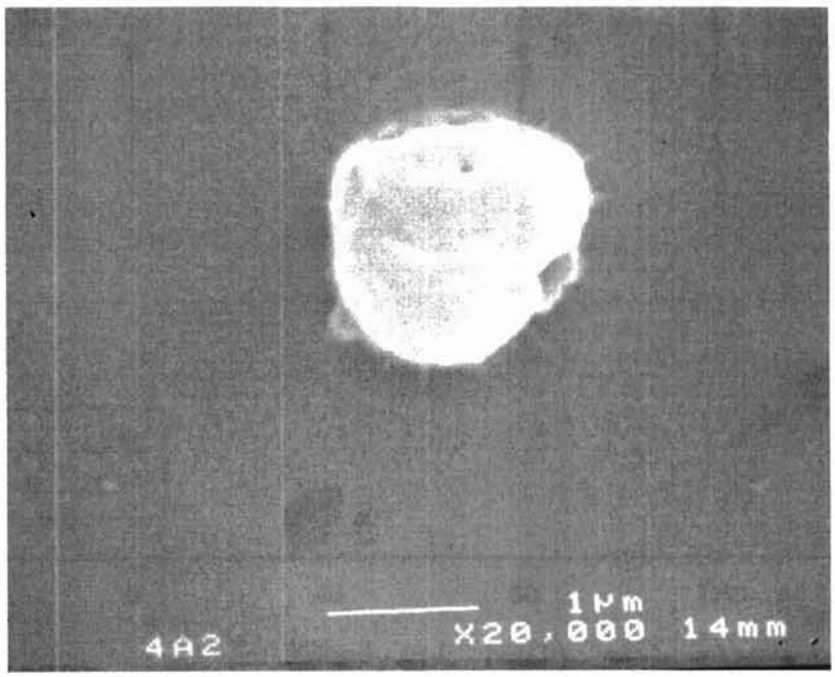

Figure 5. Scanning electron micrograph of 980625-4A.

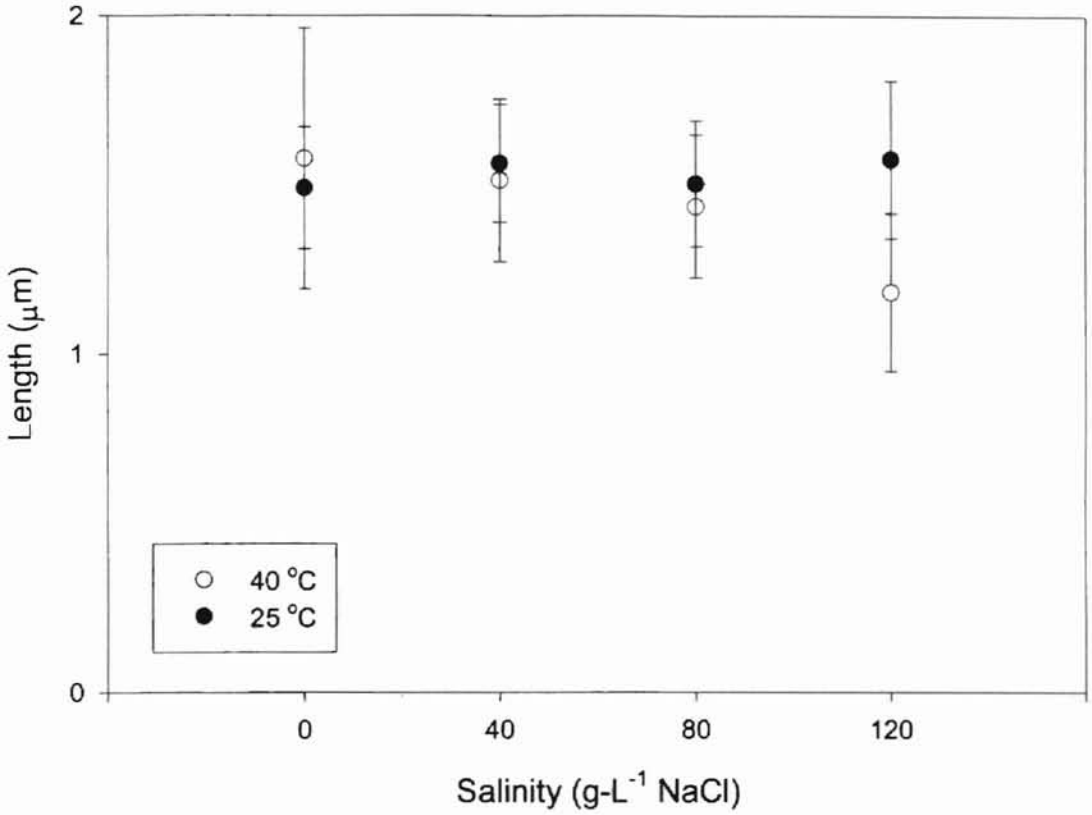

Figure 6. Major axis of 980625-4A grown at 25 and 40 °C in AS100 at different salinities. For 25 °C cultures: 0, 40, 80, 120 g-L^{-1}, n = 109, 370, 289, 201, respectively. For 40 °C cultures: 0, 40, 80, 120 g-L^{-1}, n = 152, 243, 201, 284 respectively. Mean ± SD.

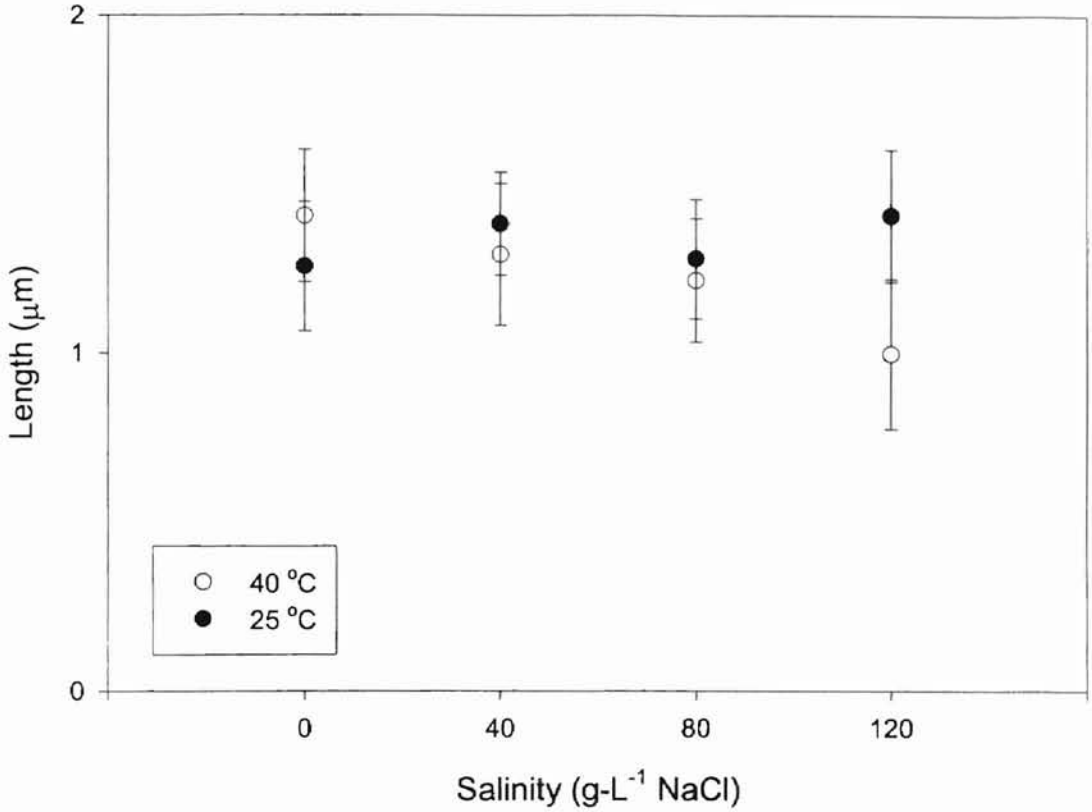

Figure 7. Minor axis of 980625-4A grown at 25 and 40 °C in AS100 at different salinities. For 25 °C cultures: 0, 40, 80, 120 g-L^{-1}, n = 109, 370, 289, 201, respectively. For 40 °C cultures: 0, 40, 80, 120 g-L^{-1}, n = 152, 243, 201, 284 respectively. Mean ± SD.

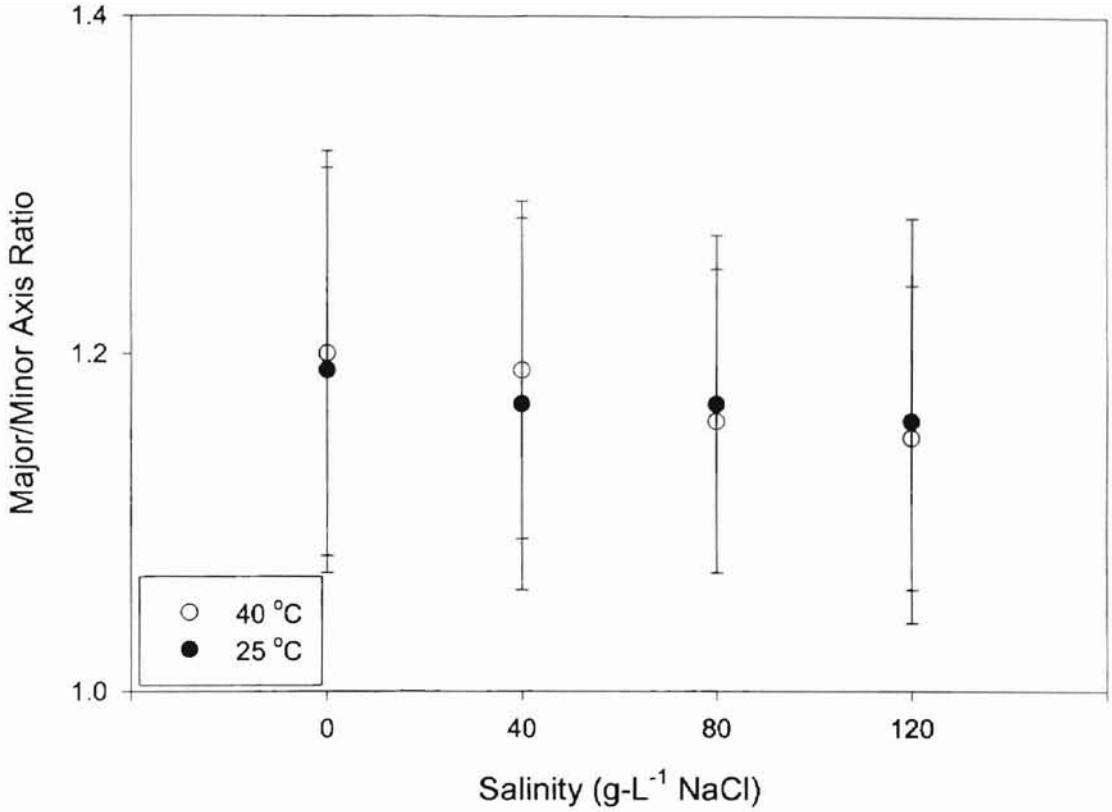

Figure 8. Ratio of major axis:minor axis of 980625-4A grown at 25 and 40 °C in AS100 at different salinities. For 25 °C cultures: 0, 40, 80, 120 g-L^{-1}, n = 109, 370, 289, 201, respectively. For 40 °C cultures: 0, 40, 80, 120 g-L^{-1}, n = 152, 243, 201, 284 respectively. Mean ± SD.

The ratio of the major:minor diameters of 980625-4A cells grown at all treatments were normally distributed (Kolmogorov-Smirnov One Sample Test using Normal distribution $P > 0.05$) (Figs. 9, 10).

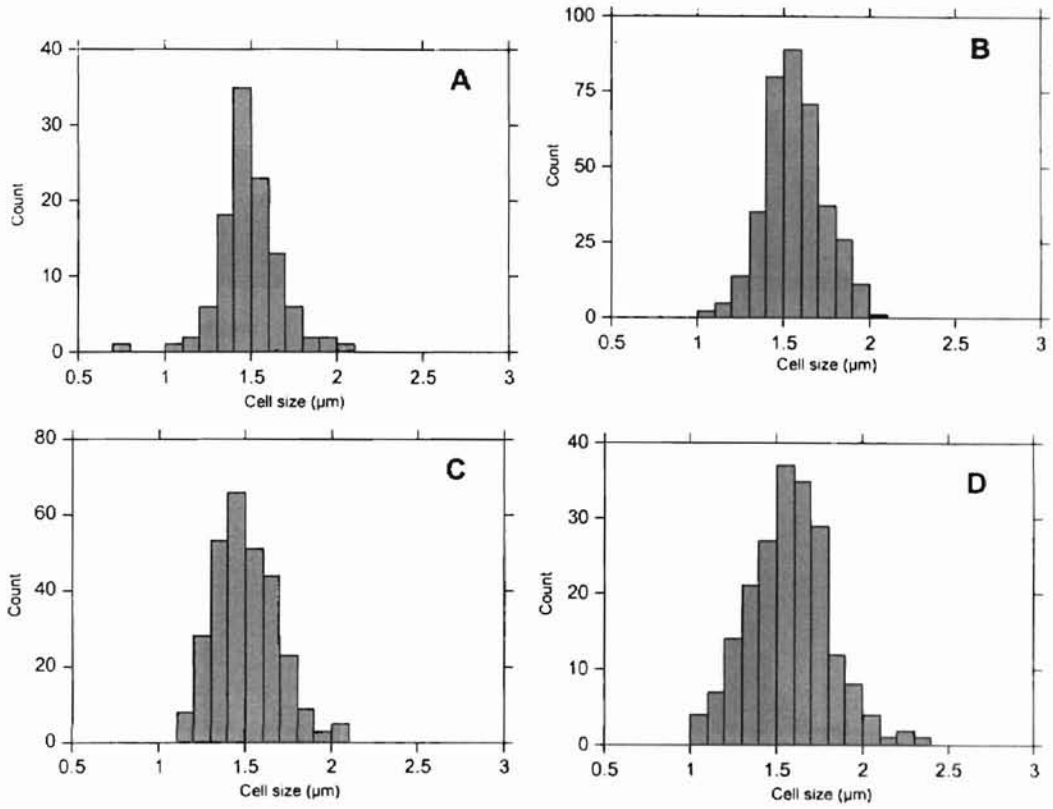

Figure 9. Distribution of major axis of 980625-4A cells grown at 25 °C in AS100 at different salinities. A = 0 g-L^{-1}, B = 40 g-L^{-1}, C = 80 g-L^{-1}, D = 120 g-L^{-1}.

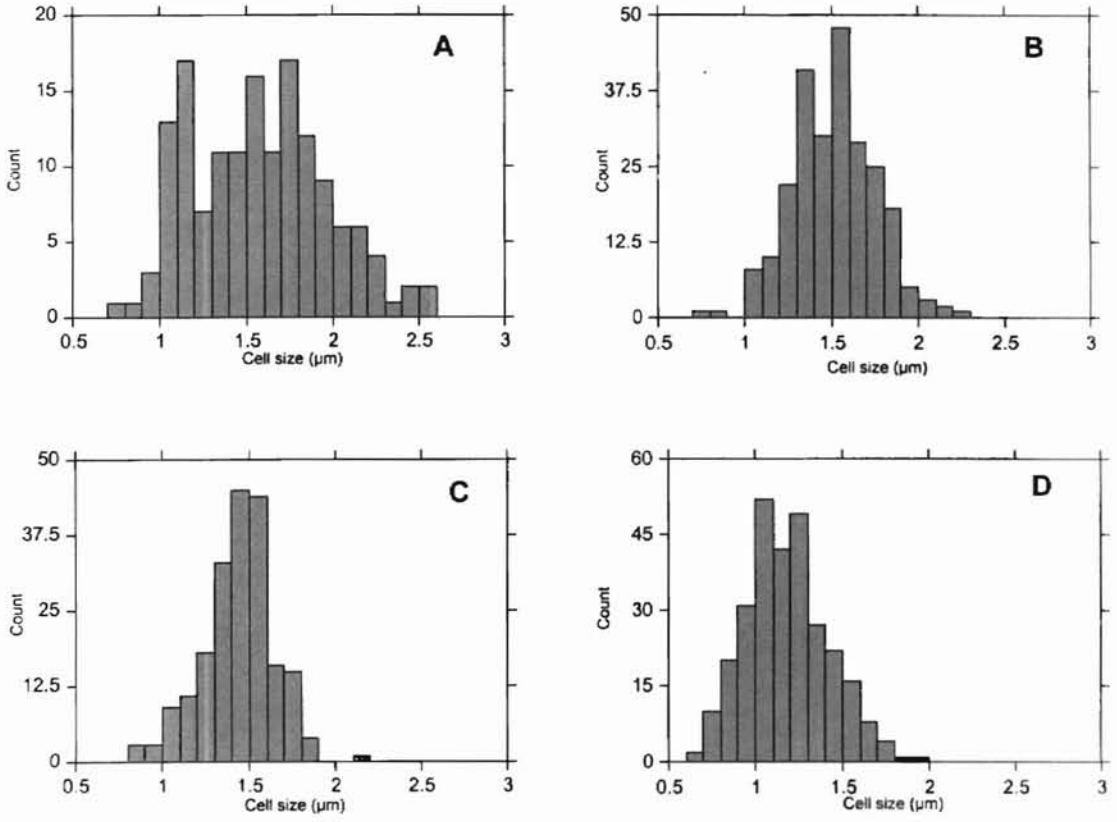

Figure 10. Distribution of major axis of 980625-4A cells grown at 40 °C in AS100 at different salinities. A = 0 g-L^{-1}, B = 40 g-L^{-1}, C = 80 g-L^{-1}, D = 120 g-L^{-1}.

B. Electron microscopy

There was no evidence of outer or vestigial structures on the cell walls in the SEM or TEM micrographs (Figs. 5, 11-16). The TEM micrographs of the 25 °C grown cells showed detailed ultrastructure (Figs. 11-15, 17-19). The 40 °C grown cells showed less ordered organelles and the chloroplast, nucleus, accumulation body were not distinguished (Figs. 20-23).

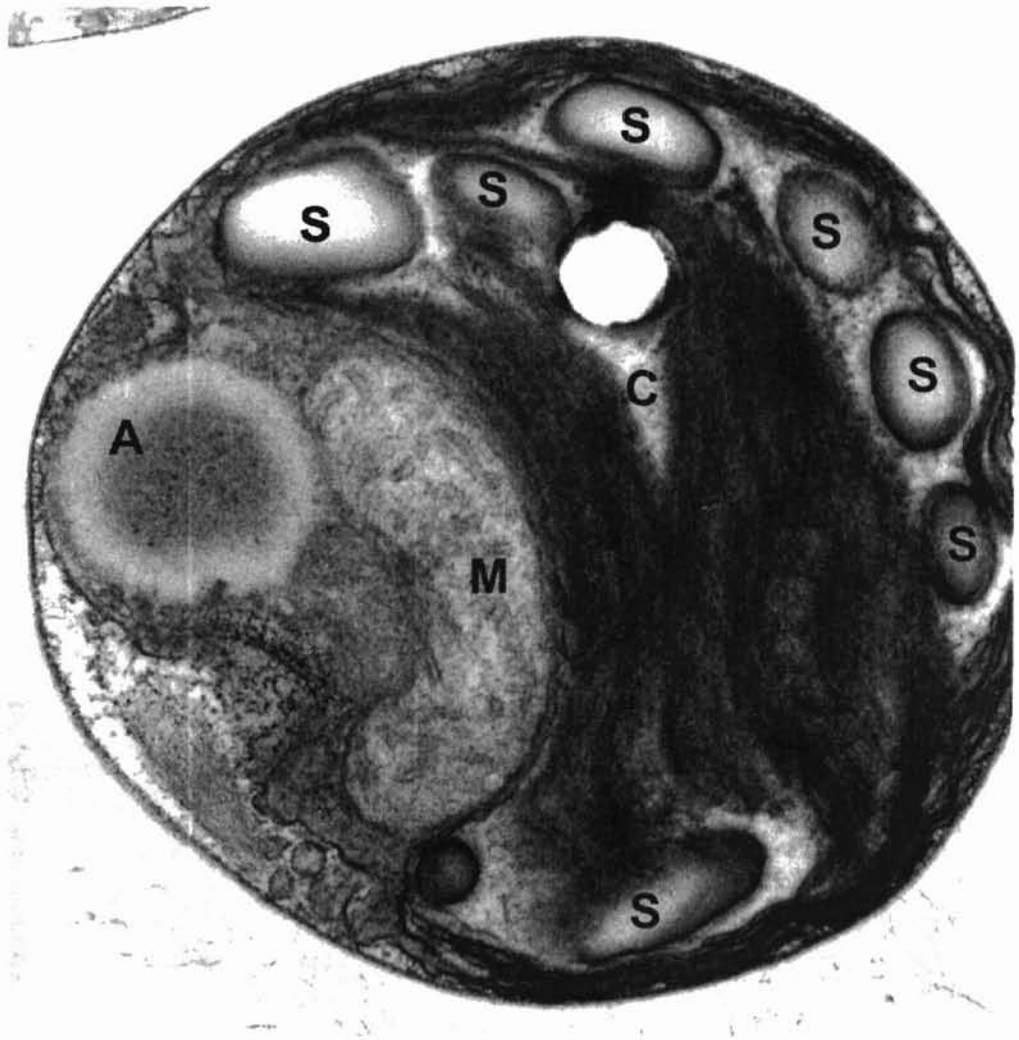

Figure 11. Transmission electron micrograph of 980625-4A cell grown at 25 °C in SP medium at 50 g-L^{-1} total dissolved solids. A=Accumulation body, M=Mitochondrion, C=Chloroplast, S=Starch grain. Enlarged 23200X.

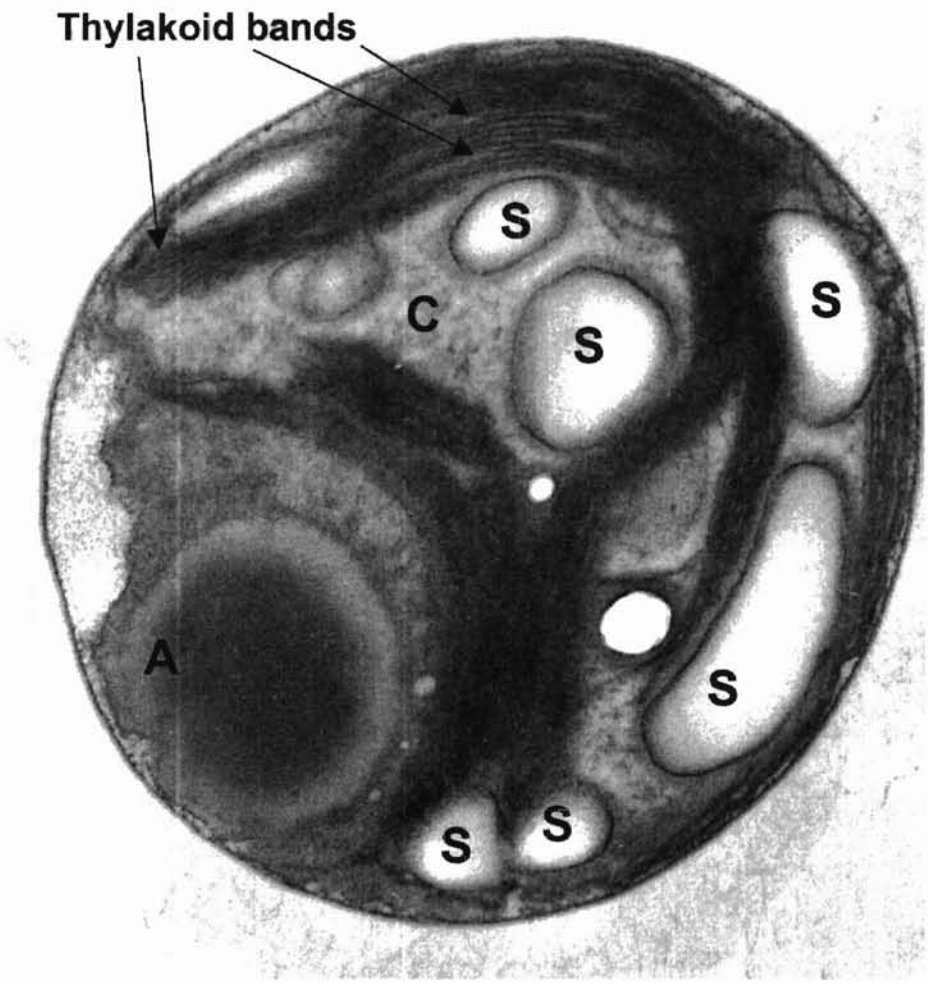

Figure 12. Transmission electron micrograph of 980625-4A cell grown at 25 °C in SP medium at 120 g-L^{-1} total dissolved solids. A=Accumulation body, C=Chloroplast, S=Starch grain. Enlarged 23200X.

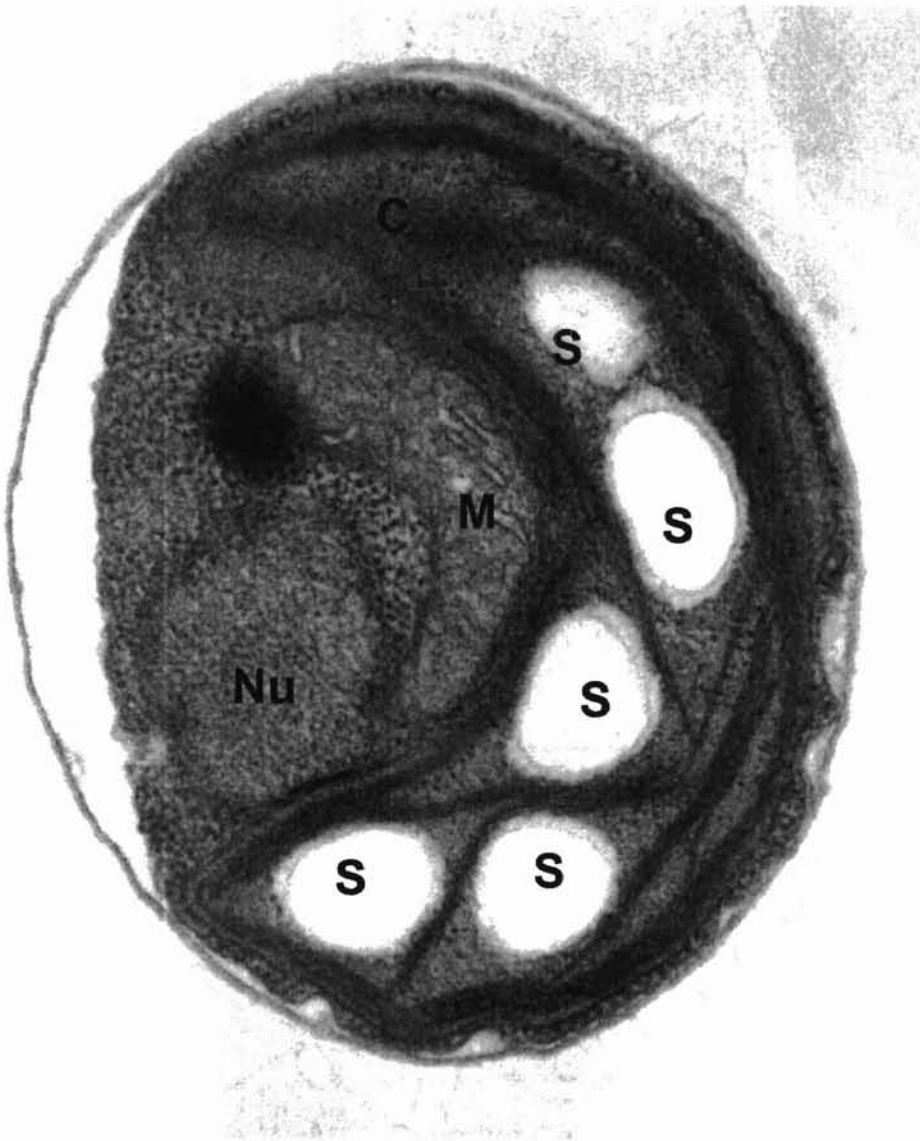

Figure 13. Transmission electron micrograph of 980625-4A cell grown at 25 °C, in AS100 medium at 0 g-L^{-1} NaCl. N=Nucleus, Nu=Nucleous, M=Mitochondrion, C=Chloroplast, S=Starch grain. Enlarged 68250X.

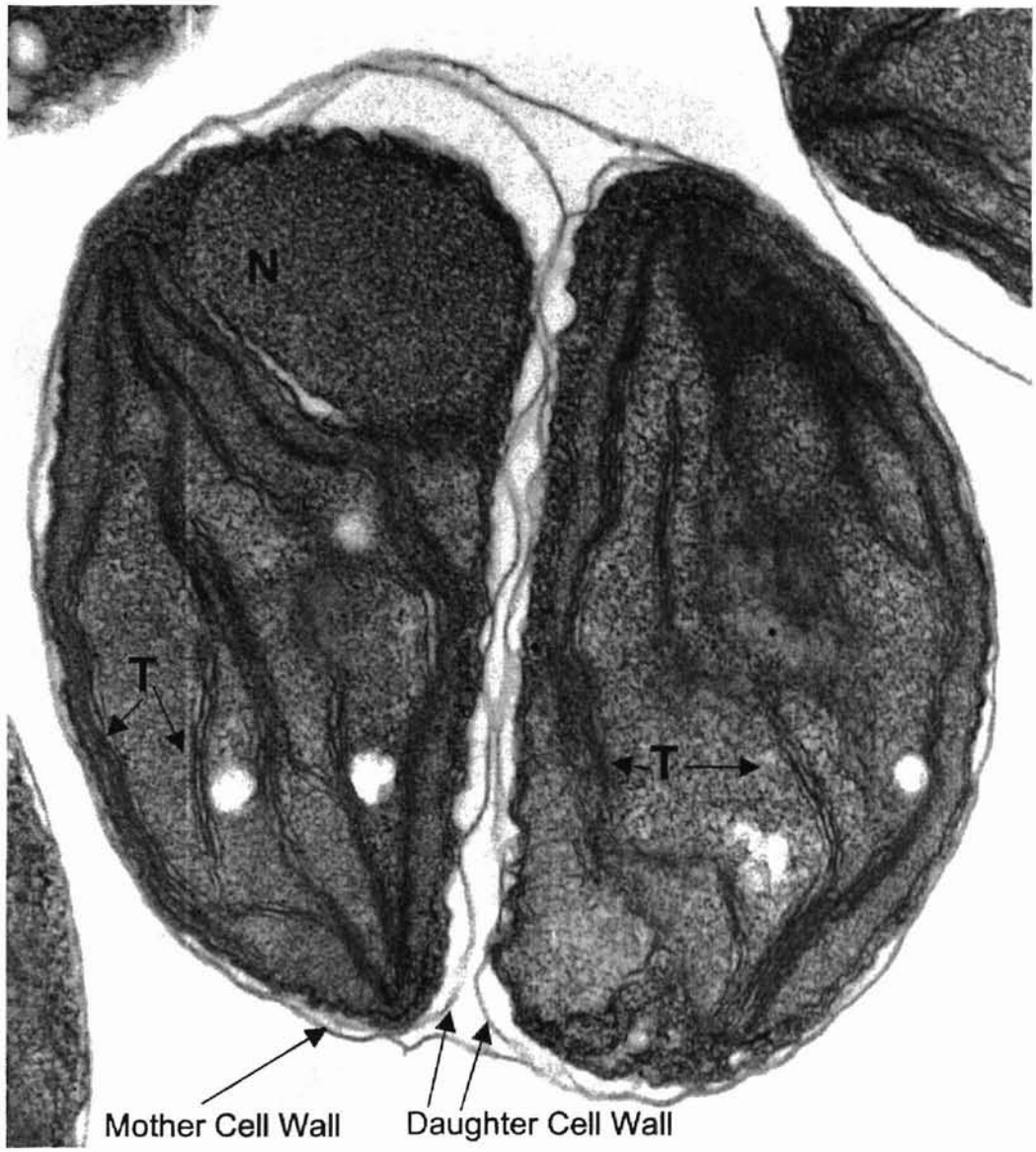

Figure 14. Transmission electron micrograph of 980625-4A cell grown at 25 °C in AS100 medium at 80 g-L^{-1} NaCl undergoing autosporulation. N=Nucleus, T=Thylakoid bands inside the chloroplast. Enlarged 67200X.

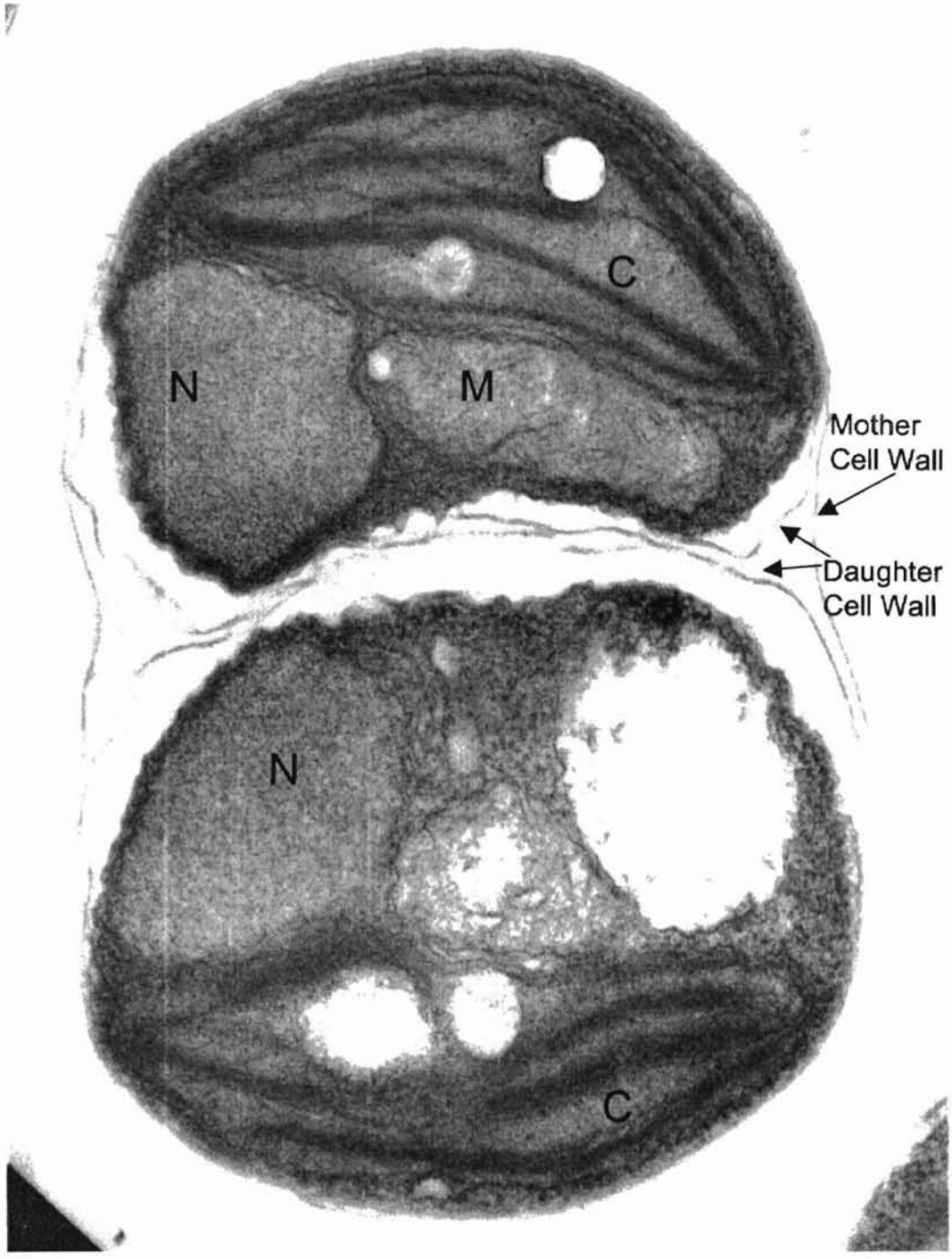

Figure 15. Transmission electron micrograph of 980625-4A cell grown at 25 °C in AS100 medium at 40 g-L^{-1} NaCl undergoing autosporulation. N=Nucleus, M=Mitochondrion, C=Chloroplast. Enlarged 75600X.

Figure 16. Scanning electron micrograph showing two cells of 980625-4A.

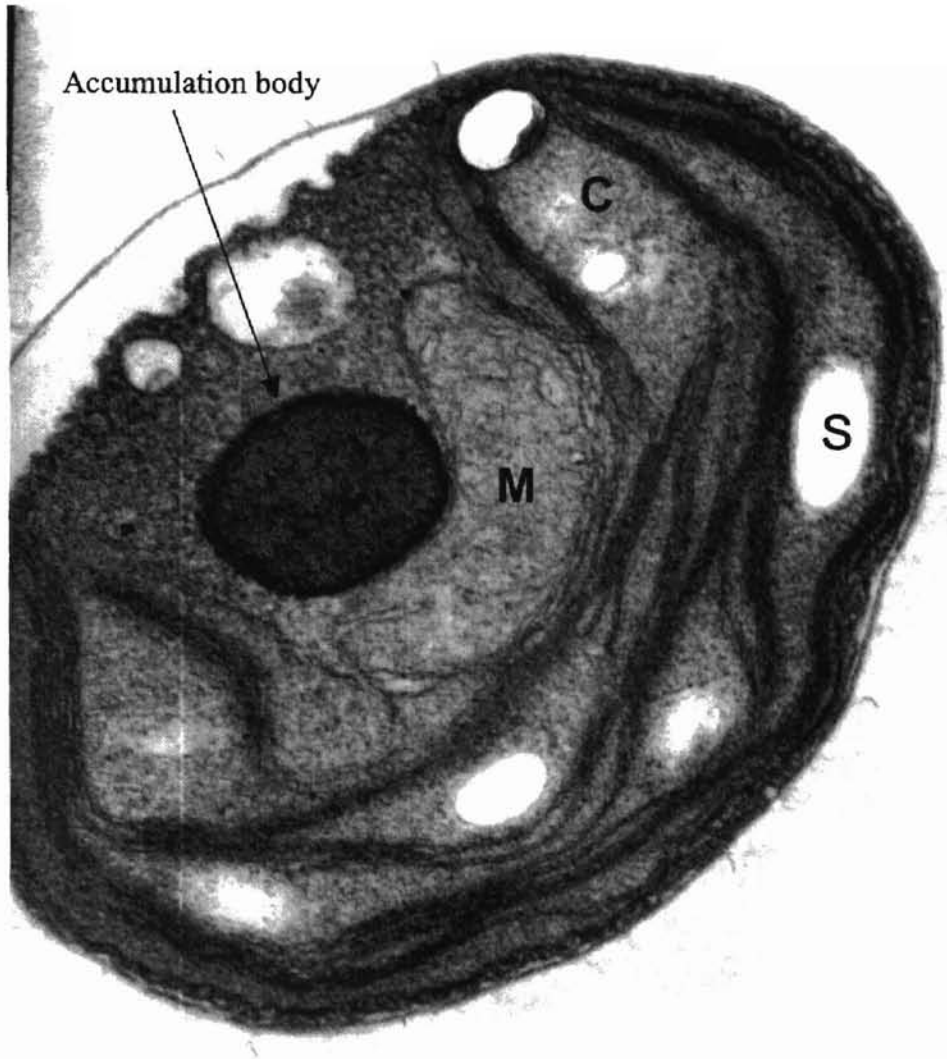

Figure 17. Transmission electron micrograph of 980625-4A cell grown at 25 °C in AS100 at 40 g-L^{-1} NaCl. M=Mitochondrion, C=Chloroplast, S=Starch grain. Enlarged 57750X.

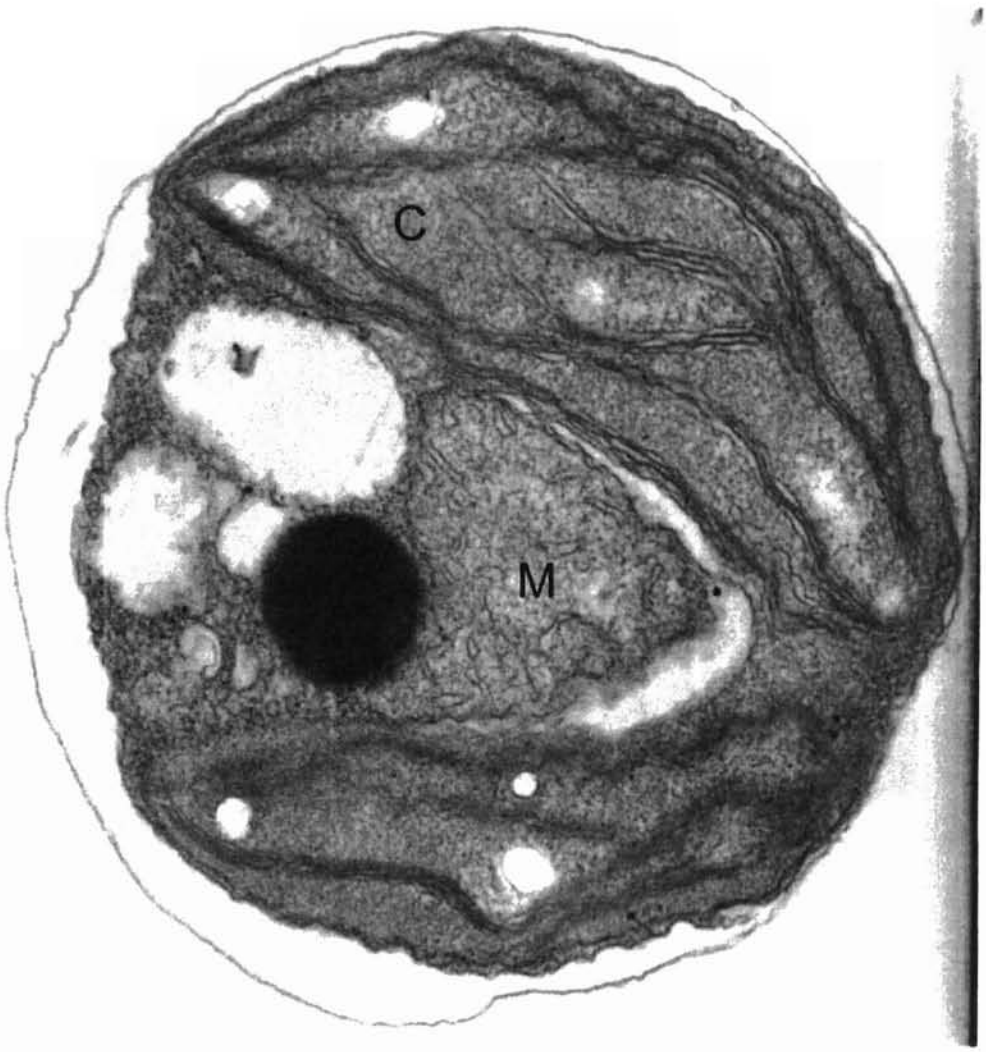

Figure 18. Transmission electron micrograph of 980625-4A cell grown at 25 °C in AS100 at 80 g-L^{-1} NaCl. M=Mitochondrion, C=Chloroplast. Enlarged 57750X.

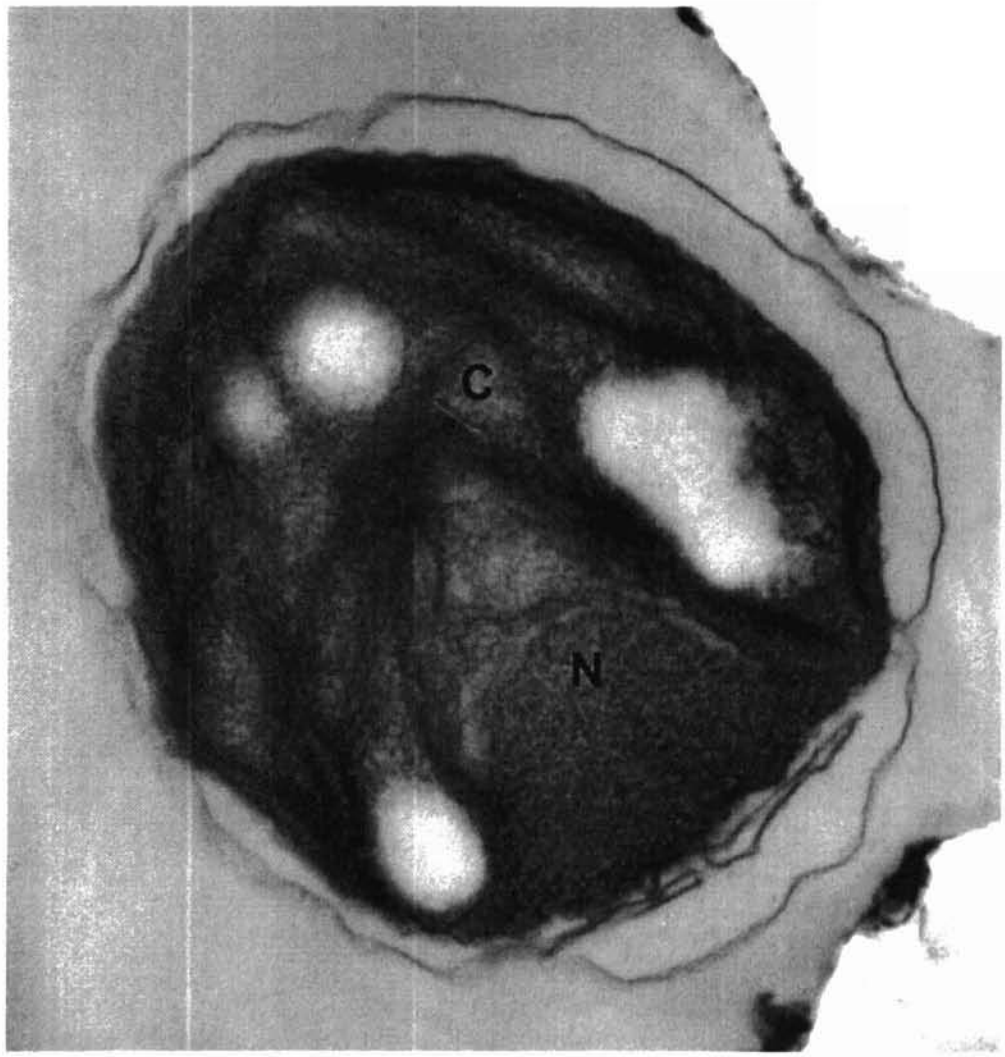

Figure 19. Transmission electron micrograph of 980625-4A cell grown at 25 °C in AS100 at 120 g-L^{-1} NaCl. N=Nucleus, C=Chloroplast. Enlarged 68250X.

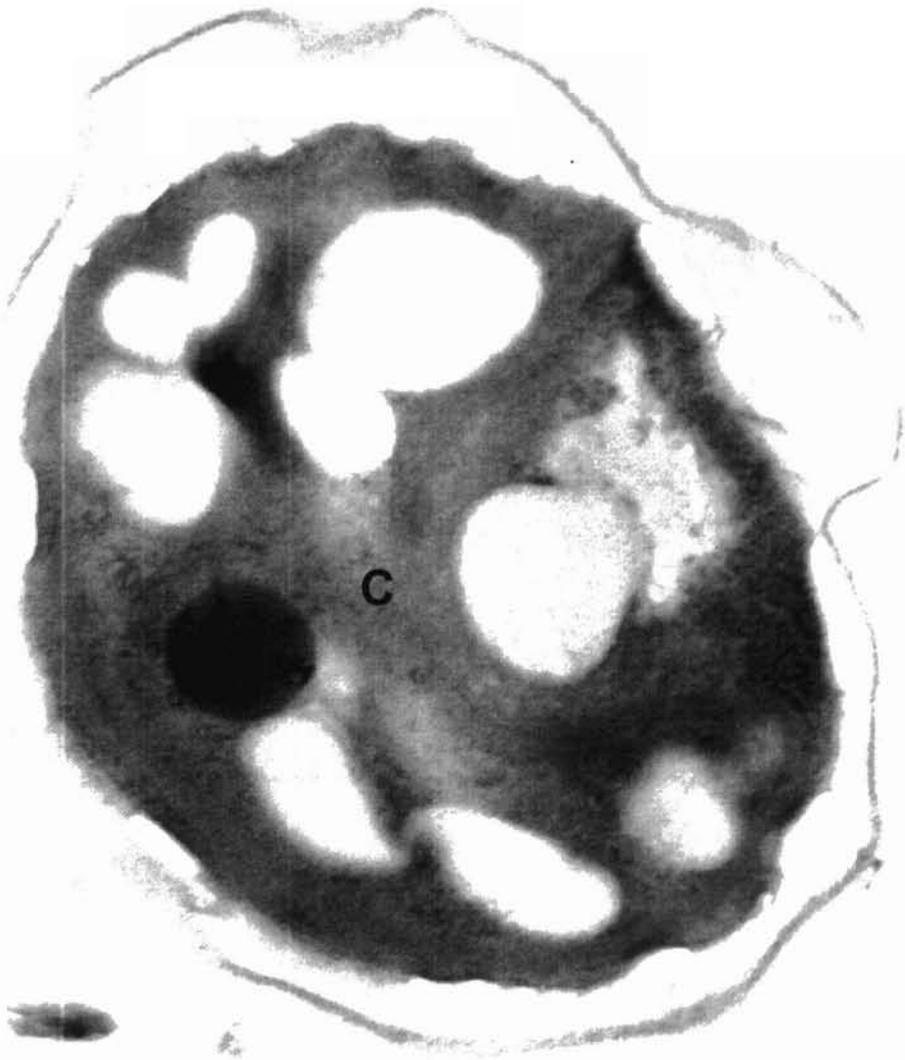

Figure 20. Transmission electron micrograph of 980625-4A cell grown at 40 °C in AS100 at 0 g-L^{-1} NaCl. C=Chloroplast. Enlarged 76125X. This cell may have been dead upon fixation.

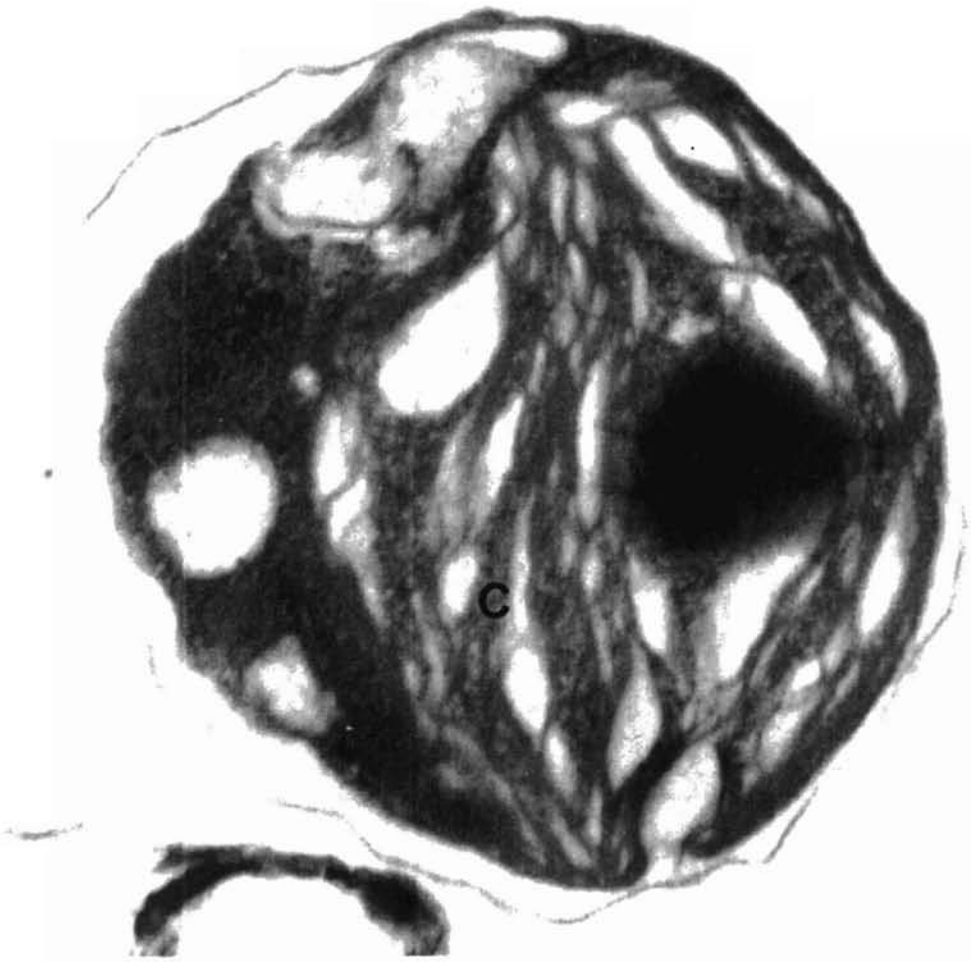

Figure 21. Transmission electron micrograph of 980625-4A cell grown at 40 °C in AS100 at 40 g-L^{-1} NaCl. C=Chloroplast. Enlarged 78750X.

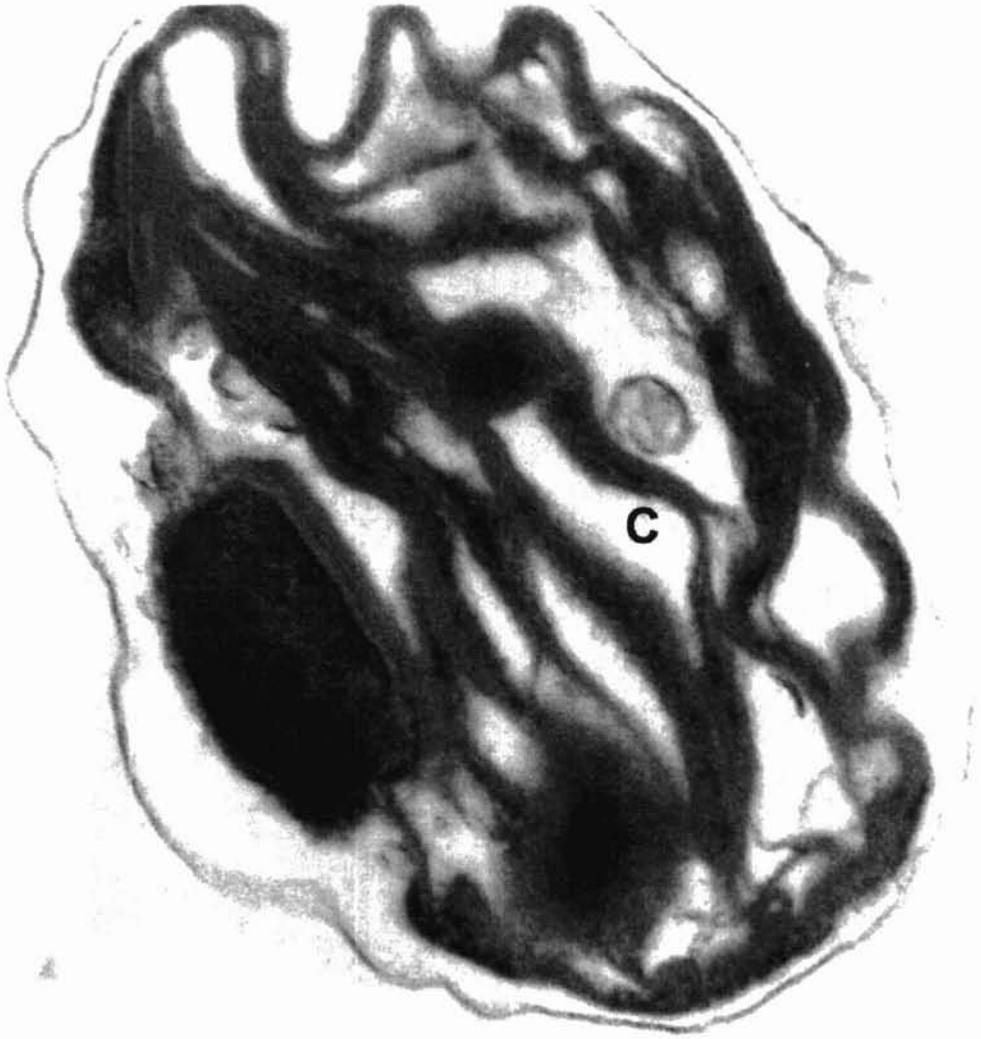

Figure 22. Transmission electron micrograph of 980625-4A cell grown at 40 °C in AS100 at 80 g-L^{-1} NaCl. C=Chloroplast. Enlarged 84000X.

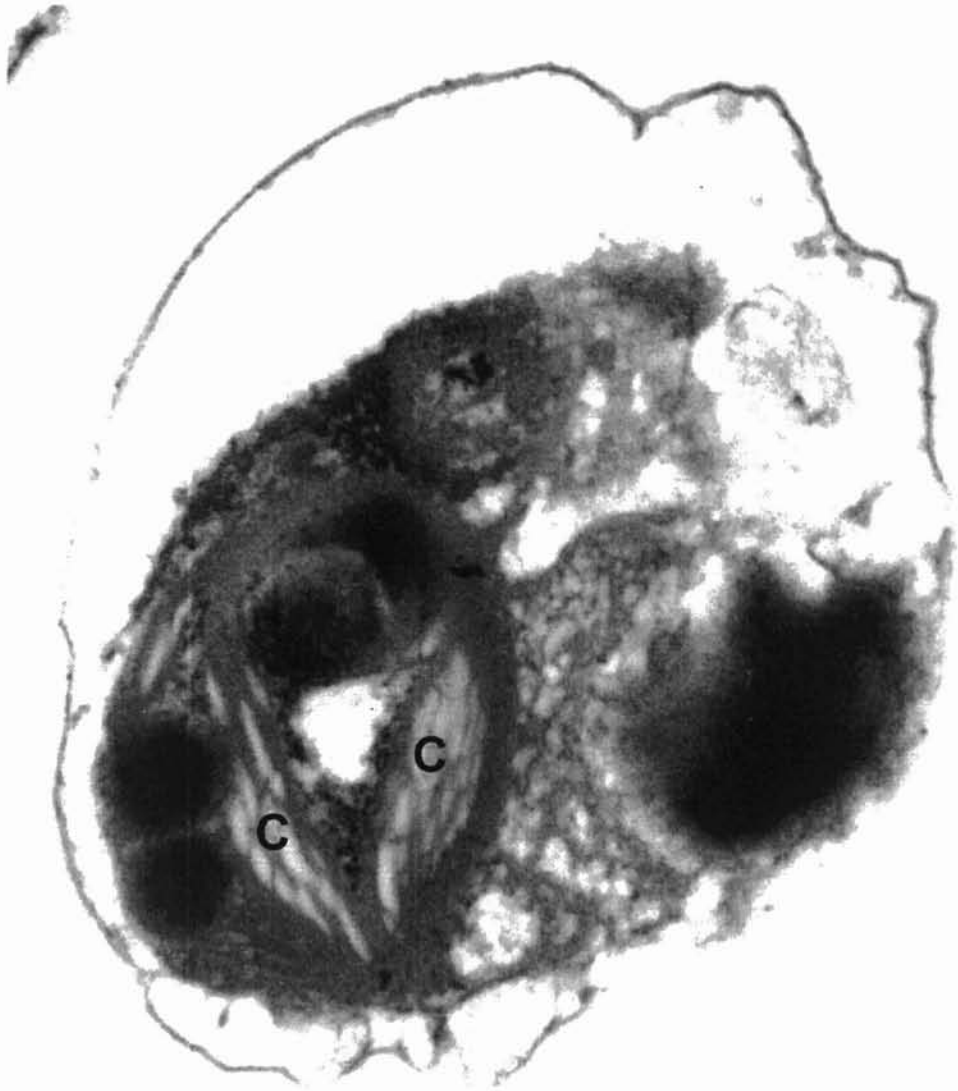

Figure 23. Transmission electron micrograph of 980625-4A cell grown at 40 °C in AS100 at 120 g-L^{-1} NaCl. C=Chloroplast. Enlarged 76125X. This cell has ruptured.

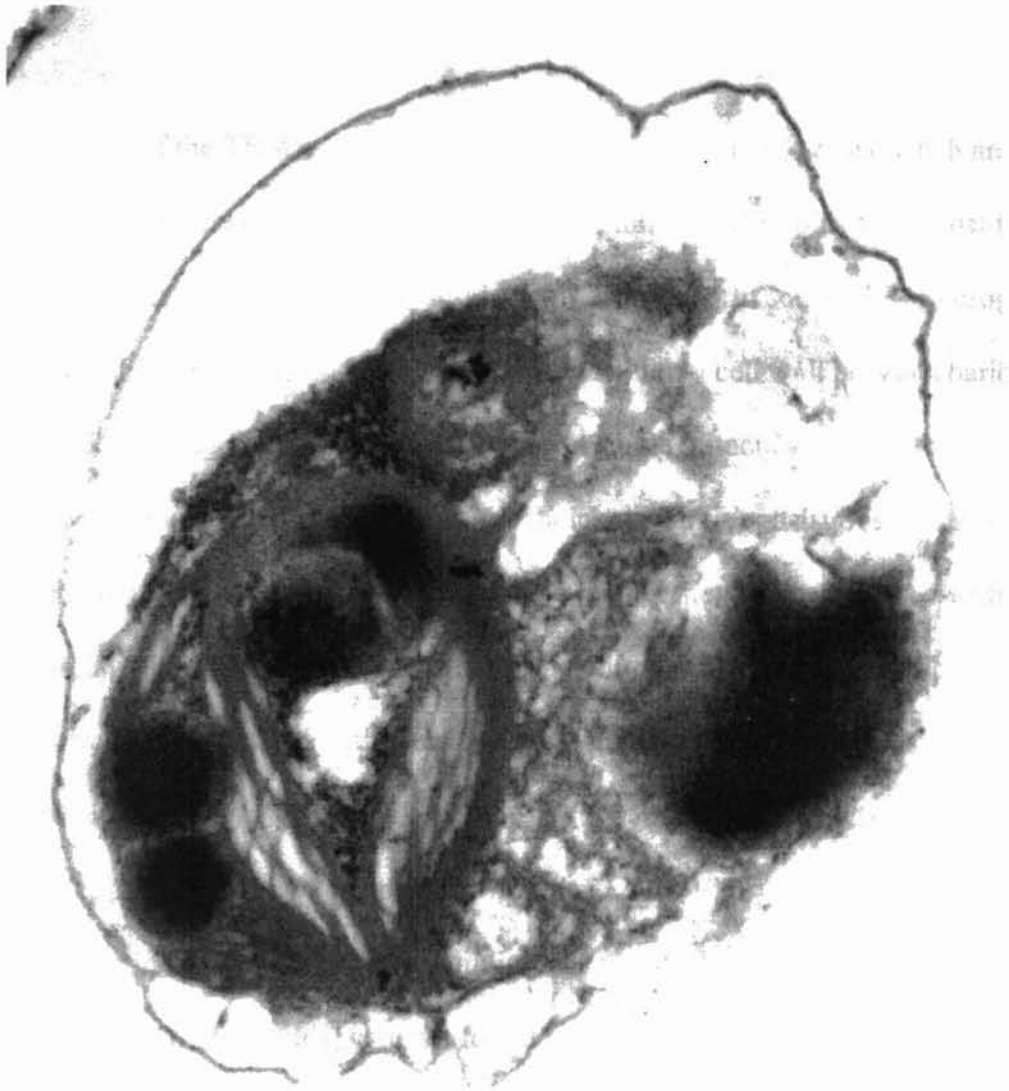

Figure 23. Transmission electron micrograph of 980625-4A cell grown at 40 °C in AS100 at 120 g-L^{-1} NaCl. C=Chloroplast. Enlarged 76125X. This cell has ruptured.

Some of the TEM micrographs of 980625-4A have black spots which are artifacts of the staining process. White grain-less areas within the chloroplasts are most likely where starch grains have fallen out during thin sectioning. In some TEM micrographs a net- or web-like area surrounds the cell and is most likely cell wall polysaccharides.

TEM micrographs of 980625-4A show a single mitochondrion with cristae and a cup-shaped chloroplast (Figs. 11, 12). The thylakoid bands consist of five stacks of thylakoid membranes (Fig. 12). The thylakoid membranes are interspersed with several oval starch grains. The round structure near the mitochondrion may be a lipid body. A similar structure associated with the chloroplast in *Nannochloropsis* sp. is referred to as an accumulation body (Fisher et al. 1998). A TEM micrograph of 980625-4A grown at AS100, 0 g-L^{-1} NaCl shows the nucleus with nucleolus (Fig. 13). The autospores have several areas in the chloroplast where individual and stacked thylakoid membranes are distinct (Fig. 14). Pyrenoids were not found in any TEM micrographs of 980625-4A. TEM micrographs also failed to show evidence of vacuoles in 980625-4A.

Autosporulation has been documented in 980625-4A cultured at 25 °C in AS100 at 0, 40, 80, 120 g-L^{-1} NaCl and at 40 °C in 40, 80, 120 g-L^{-1} NaCl. It was not possible to document autosporulation in cells grown at 40 °C in AS100, 0 g-L^{-1} NaCl because of poor quality TEM thin sections. Two TEM micrographs were chosen as representatives of the micrographs depicting autosporulation (Figs. 14, 15). In both TEM micrographs of 980625-4A, the parent cell wall is distinct and separate from the cell walls of the two

daughter cells. In a few areas where the protoplast of the daughter cell has separated from the cell wall, two distinct cell walls can be seen. A SEM micrograph shows two cells within a casing though this micrograph cannot be used as documentation of autosporulation because the autospore cell wall cannot be determined in SEM micrographs (Fig. 16).

One TEM micrograph from each of the 980625-4A cultures: AS100 at 0, 40, 80, 120 g-L^{-1} NaCl at 25 °C and AS100 at 0, 40, 80, 120 g-L^{-1} NaCl at 40 °C was visually analyzed for salinity- and temperature-dependent ultrastructure changes (Figs. 13, 17-23). The micrographs of cells grown in the three lower salinities at 25 °C show the mitochondrion with distinct cristae, cup-shaped chloroplast with thylakoid membranes, and several starch grains. The TEM micrographs of the 40 °C grown cells show highly distorted cells, some may have been dead upon fixation (Figs. 20-23). Mitochondria were not recognizable in many of the 40 °C cultures. The micrographs of cells grown at 40 °C and 40, 80, 120 g-L^{-1} salinity showed distorted chloroplasts with rope-like thylakoids. Neither the chloroplast nor mitochondrion was evident in micrographs of cells grown at 40 °C in 0 g-L^{-1} salinity. Cellular membranes also showed evidence of distortion for all cells grown at 40 °C, and cell walls were undulate and expanded at 80 g-L^{-1} salinity and to a greater degree at 120 g-L^{-1} NaCl.

C. Growth of cultures grown at 25 and 40 °C in different salinities

The growth of 980625-4A at 25 and 40 °C in AS100 in 0, 40, 80,120 g-L^{-1} NaCl was monitored by calculating cell densities from A_{750} measurements (Figs. 24, 25). Cell densities for the period from day 2 through day 6 were used to calculate divisions per day

for all treatments. The culture grown at 40 °C in AS100 at 0 g-L^{-1} NaCl showed little evidence of growth during the experiment. The growth rates of 980625-4A were higher at 25 °C for all salinities, and were highest at 40 g-L^{-1} NaCl for both temperatures (Fig. 26). The coefficient of determination (r^2) exceeded 0.9 for all cultures except 25 °C at 80 and 120 g-L^{-1} NaCl and 40 °C at 0 g-L^{-1} NaCl. Two-Way ANOVA analysis of these results showed significant main effects of temperature, salinity, and salinity-temperature interaction on growth rates (P = 0.000, 0.019, and 0.019, respectively).

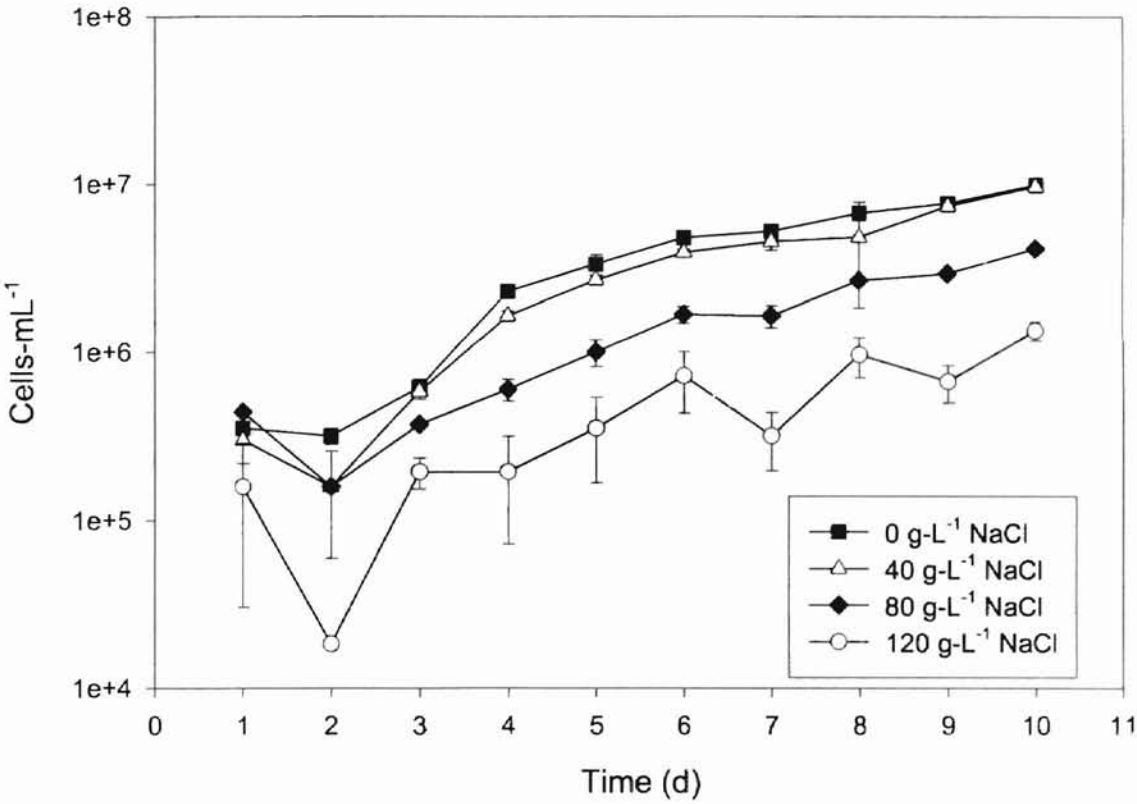

Figure 24. Growth curves for 980625-4A grown at 25 °C in AS100 medium at different salinities, Mean ± SD, n = 4.

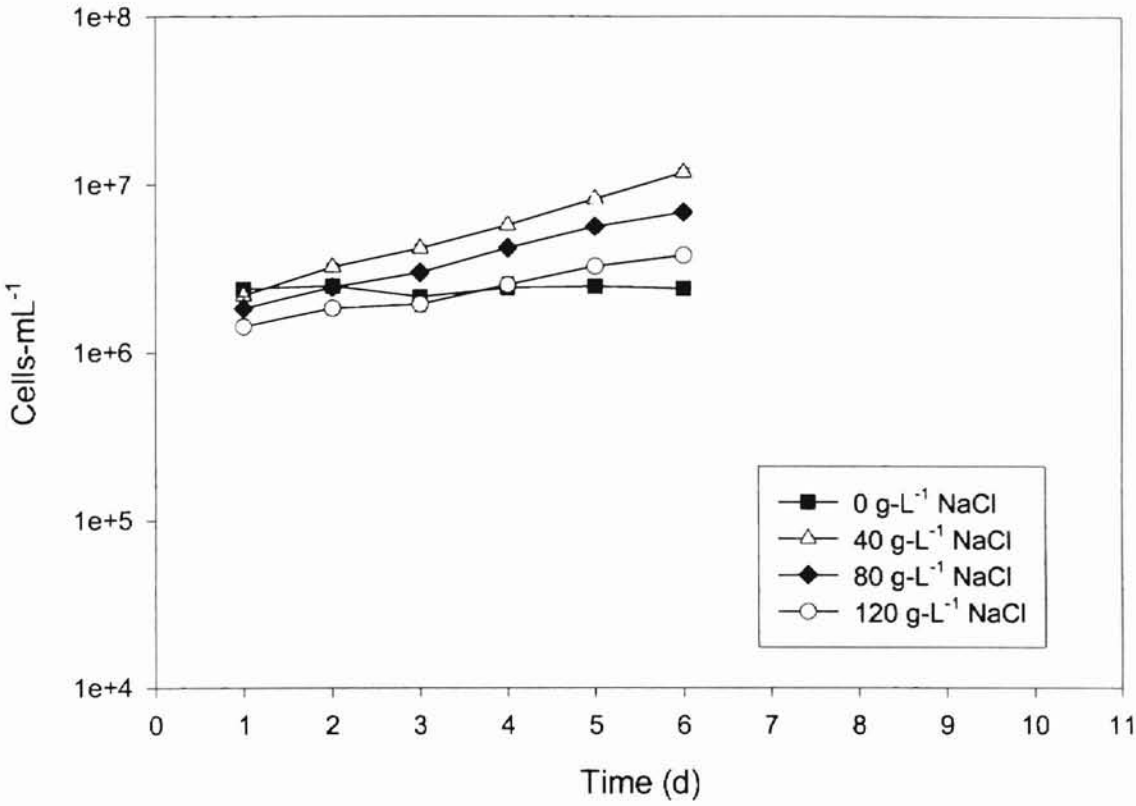

Figure 25. Growth curves for 980625-4A grown at 40 °C in AS100 medium at different salinities, Mean ± SD, SD error bars are smaller than the symbol, n = 4.

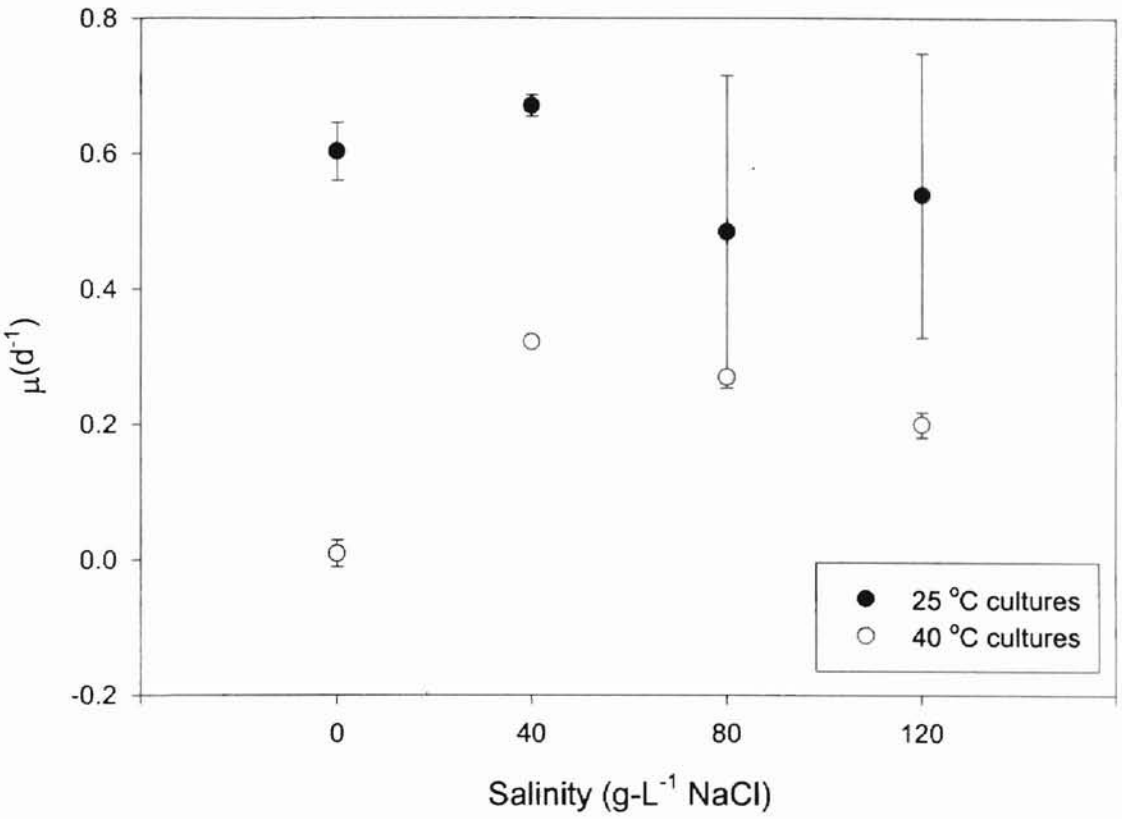

Figure 26. Specific growth rates for 980625-4A grown at 25 and 40 °C in AS100 medium at different salinities, Mean ± SD, n = 4.

D. Ruthenium red staining

Ruthenium red is a dye that adheres to acidic polymers (pectin) of the cell walls in some algae and is used as a minor taxonomic marker in classification. After the ruthenium red treatment, the algal species tested retained different amounts of the dye (Table 2). The *Chlorella* spp. retained more ruthenium dye when compared with the *Nannochloris* spp. and 980625-4A as evident in the color of the ruthenium red and AS100 supernatants. The color of the ruthenium red supernatant for *Nannochloris* spp.

and 980625-4A was close to the original color of the ruthenium dye indicating the ruthenium red dye did not stain the cell walls. Therefore, there is very little acidic polymer in these algae. The colors of the ruthenium red supernatants of the *Chlorella* spp. were lighter than the original color of the ruthenium red dye indicating all *Chlorella* spp. tested contained acidic polymers in their cell walls.

Table 2. Ruthenium red staining of microalgae.

Supernatant: Alga	Ruthenium Red	First Wash	Second Wash
980625-4A	4	1	1
Nannochloris sp. UTEX 2378	4	2	1
Nannochloris sp. UTEX 2379	4	1	1
Chlorella vulgaris UTEX 1809	2	2	3
Chlorella saccharophila UTEX 2469	1	3	2
Chlorella sorokiniana UTEX 1810	1	3	3

Key to intensity of stain remaining in supernatant: 4 = original ruthenium red solution, 3 = darkest pink, 2 = medium pink, 1 = lightest pink.

E. Pigments

Pigment analysis was performed using HPLC on 980625-4A, *Nannochloris* sp. UTEX 2378, *Nannochloris* sp. UTEX 2379, *Chlorella vulgaris* UTEX 1809, *C. sorokiniana* UTEX 1810, and *C. saccharophila* UTEX 2469. Chlorophyll *b* was found in all algae tested except *Nannochloris* UTEX 2379 (Figs. 27, 28). The major carotenoids in 980625-4A are lutein (C7) followed by violaxanthin (C3), neoxanthin (C2), and β-carotene (C11). Lesser amounts of astaxanthin (C10) and vaucheriaxanthin ester (C8) were also observed (Table 3). Both Chl *a* and Chl *b* appeared as double peaks which had indistinguishable UV-visible spectra. The minor peaks are attributed to isomeric forms of each chlorophyll.

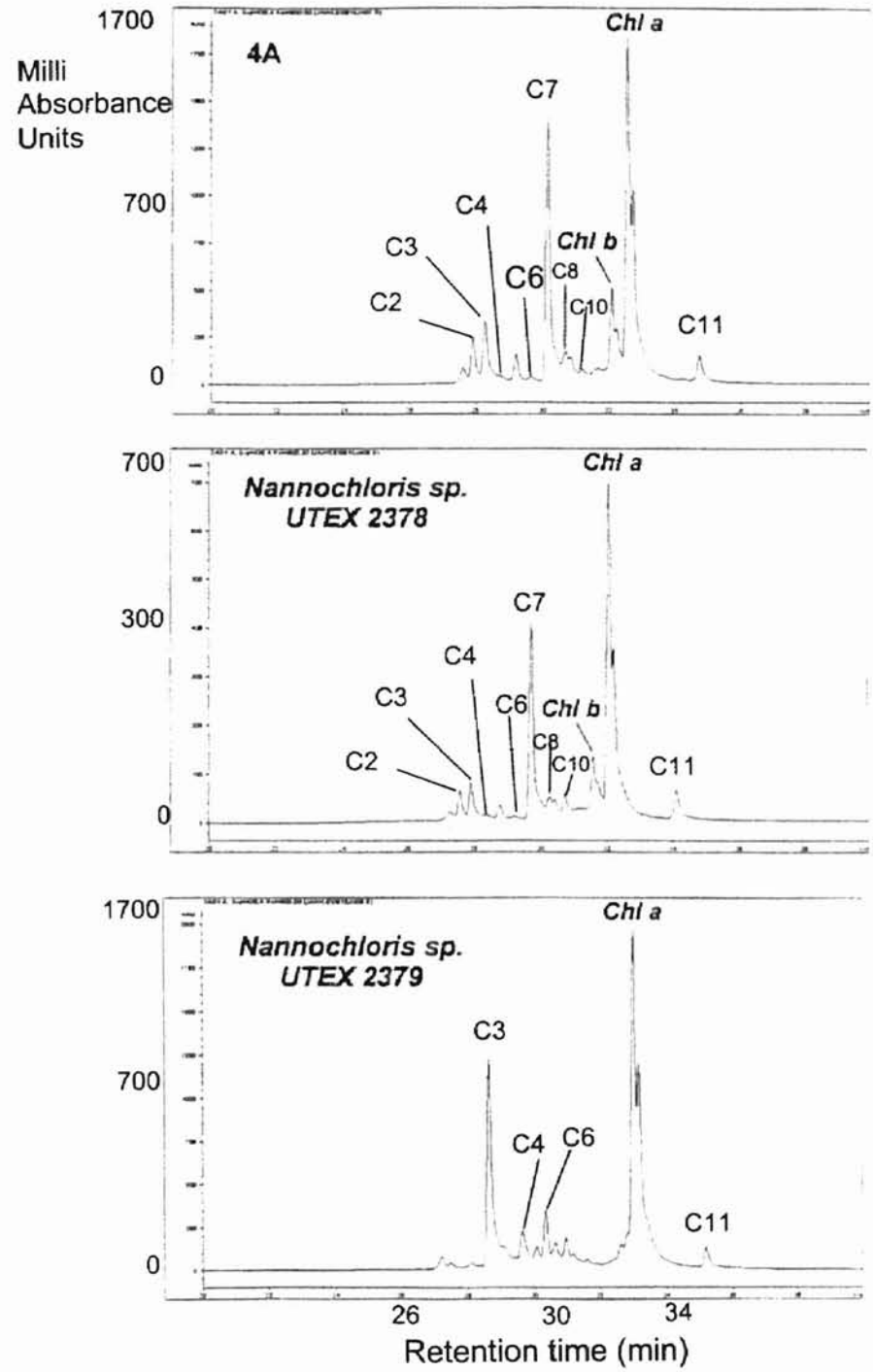

Figure 27. HPLC chromatograms for $\lambda = 436$ nm of extracts from 980625-4A (upper trace) and Nannochloris spp. UTEX 2378 (middle trace) and UTEX 2379 (lower trace).

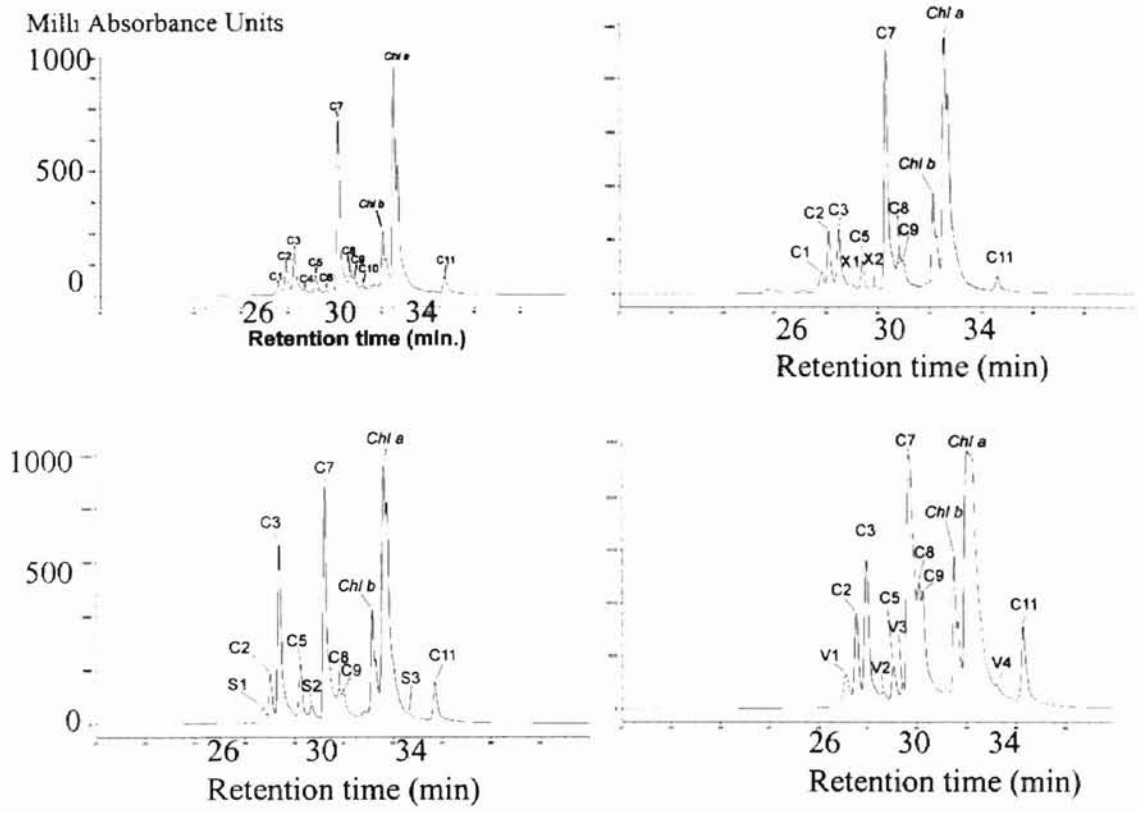

Figure 28. HPLC chromatograms for $\lambda = 436$ nm of extracts from 980625-4A (upper left) and *Chlorella saccharophila* UTEX 2469 (upper right), *C. sorokiniana* UTEX 1810 (lower left), and *C. vulgaris* UTEX 1809 (lower right).

Table 3. Carotenoids extracted from 980625-4A grown at 25 °C in AS100 at 40 g-L^{-1} NaCl as analyzed by HPLC with diode array detection.

Identification Number	Retention time (min.)	Assignment	Absorbance maxima (nm) in HPLC solvent
C1	27.595	unknown	481, 444, 473*
C2	27.901	Neoxanthin	414, 439*, 468
C3	28.269	Violaxanthin	418, 442, 472*
C4	28.719	unknown	423, 448*, 472
C5	29.179	unknown	447*, 475
C6	29.621	unknown	412, 437*, 465
C7	30.135	Lutein	449*, 475
C8	30.689	Vaucheriaxanthin ester	419, 444*, 472
C9	30.843	unknown	420, 440*, 469
C10	31.185	Astaxanthin	482*
C11	34.727	β-Carotene	456*, 484

*indicates wavelength of strongest absorbance in the region of the spectrum from 300-900 nm.

F. Osmolytes

The solutes proline, glycerol and glucose (as total hexose and presumably mostly glucose) were observed to be the most abundant constituents identified in perchloric acid extracts of 980625-4A cultured at 25 and 40 °C (Figs. 29, 30). Concentrations of all three of these compounds increased in 980625-4A cells grown in the higher salinity, especially proline, which increased about tenfold (Table 4). Glucosylglycerol and several amino acids (glutamate, glycine) were also detected but in low abundance relative to proline. Trace amounts of glycine betaine and ectoine were observed, but levels were less than 0.1 % of the amount of proline.

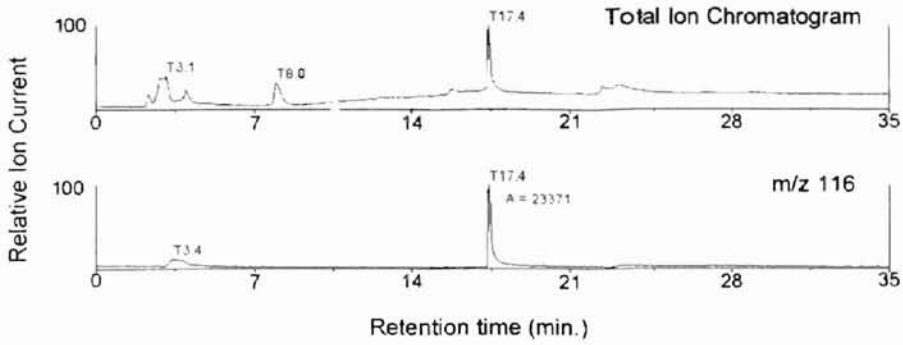

Figure 29. Total ion chromatogram and reconstructed ion chromatogram for *m/z* 116 (proline) for perchloric acid extract of 980625-4A grown at 25 °C in AS100 at 0 g-L^{-1} NaCl.

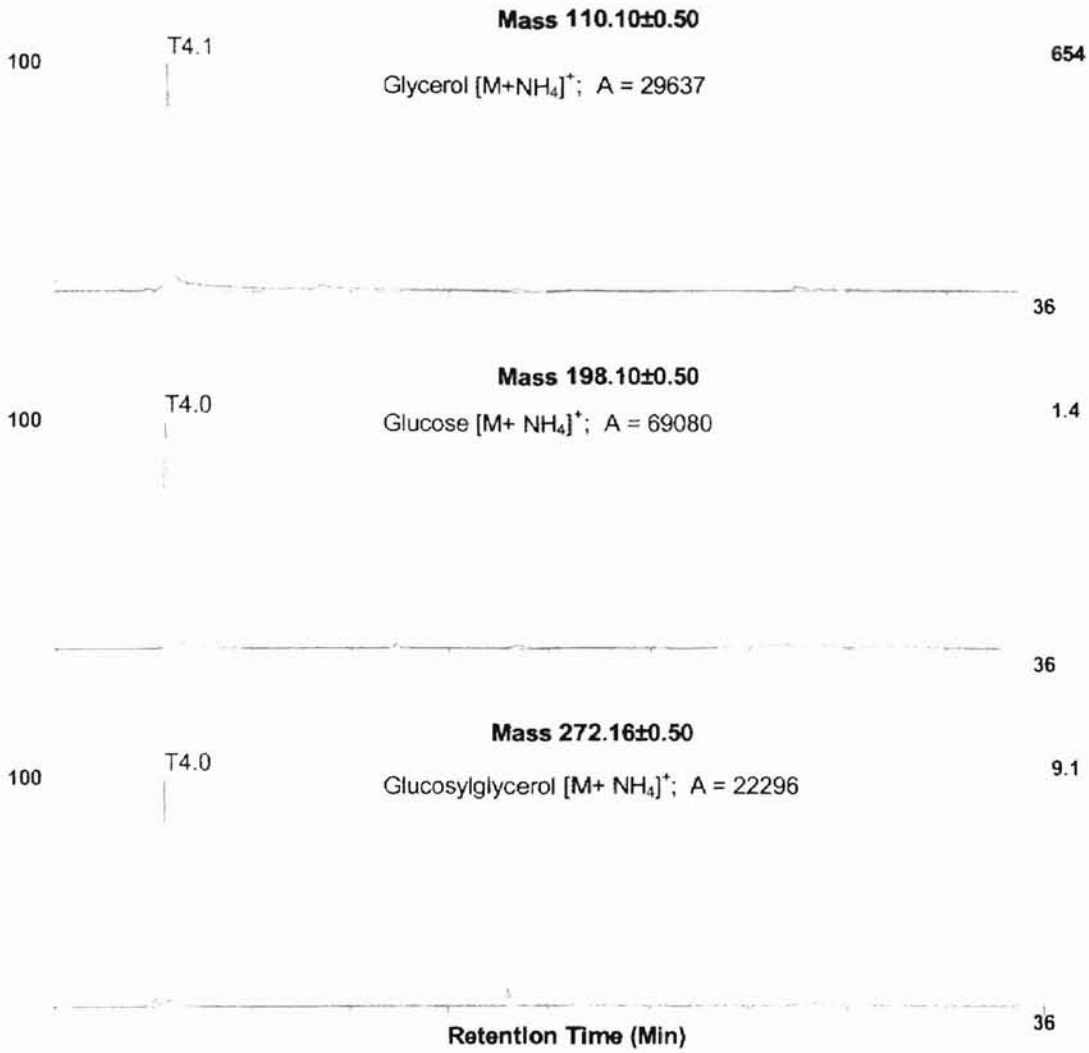

Figure 30. Reconstructed ion chromatograms for m/z 110 (glycerol), 198 (glucose), and 272 (glycosylglycerol) of perchloric acid extracts of 980625-4A grown at 25 °C in AS100 at 40 g-L^{-1} NaCl.

Table 4. Difference in osmolyte reconstructed ion chromatogram peak areas determined using HPLC-MS between perchloric acid extracts of 980625-4A grown at 25 °C in AS100 at 0 and 40 g-L^{-1} NaCl. Values normalized to absorbance at 750 nm.

Compound	µg osmolyte-mL^{-1} of culture (0 g-L^{-1} NaCl)	µg osmolyte-mL^{-1} of culture (40 g-L^{-1} NaCl)	Δ%
Proline	1.11	15.86	+1329
Glycerol	3.54	6.73	+90
Glucose	8.00	19.23	+140
Glucosylglycerol	4.43	6.21	+40

DISCUSSION

A. Range of environmental conditions that support growth of 980625-4A

980625-4A was collected from the SPNWR and, because it tolerated different salinity and cold stresses, further physiological experiments were planned. Identification of the alga is necessary to place it within the correct taxonomic category. 980625-4A will be compared to described coccoid green algae, particularly the morphologically similar genera *Nannochloris* and *Chlorella* and model halophilic genus *Dunaliella*.

980625-4A was maintained in the laboratory in SP medium supplemented with f/2 nutrients (Guillard and Ryther 1962). SP medium is prepared from salt crystals collected from the SPNWR at different places and times of the year and dissolved in distilled water to the required salinity. Consistency in medium composition would be impossible to maintain over prolonged periods using salts collected from the SPNWR. Therefore, AS100 was chosen for the experimental defined medium because it is an artificial sea water medium specially modified to culture L1644 *Dunaliella* at the University of Texas at Austin Algal Collection. The NaCl concentration of AS100 can

be adjusted for experiments without altering other nutrient concentrations, and the medium allowed lush growth of 980625-4A. A defined medium is important to use when measuring physiological parameters, such as division rates, cell size, pigment composition, because these may be nutrient dependent.

The range of environmental conditions an organism can tolerate has been used as a taxonomic indicator (Kessler and Huss 1992). The maximum growth temperature was investigated in seventeen *Chlorella* taxa and only *C. sorokiniana* was able to grow at 40 °C with a maximum growth temperature of 42 °C (Kessler and Huss 1992). A *Dunaliella* sp. collected from the Great Salt Lake, Utah exhibited the highest growth rates in the range of about 27-35 °C and the growth rates decreased with temperatures above and below that range. The lowest growth rate for this alga was one doubling in 23 days at 5 °C (Van Auken and McNulty 1973).

980625-4A grows well in AS100 with no added NaCl and in SP and AS100 with NaCl concentrations as high as 140 $g\text{-}L^{-1}$ indicating the alga is halotolerant. Other halotolerant halophytes, including the marine *Nannochloris eucaryota* (or *Nannochloris eucaryotum* as named by Menzel and Wild 1989), can grow well from 2 to 120 $g\text{-}L^{-1}$ total dissolved solids (Geisert et al. 1987, Tschermak-Woess 1999). *Nannochloris bacillaris*, isolated from the Indian Ocean, can grow in 0-105 $g\text{-}L^{-1}$ NaCl. The marine chlorophyte flagellate *Platymonas* sp. can grow in 2-35 $g\text{-}L^{-1}$ NaCl (McLachlan 1961). Seventeen strains of *Chlorella* were investigated for their maximum salinity growth limit. Only *C. saccharophila* was able to live in 60 $g\text{-}L^{-1}$ NaCl (Kessler and Huss 1992). The salinity range, 15-90 $g\text{-}L^{-1}$ NaCl capable of supporting growth of *Chlorococcum littorale,* a marine coccoid green alga, is narrower than that of 980625-4A and, unlike 980625-4A,

C. littorale did not survive 0 g-L^{-1} NaCl (Chihara et al. 1994). 980625-4A is able to grow at higher salinities than any of the halophilic and halotolerant algae listed above. 980625-4A is also unusual in its ability to tolerate culture temperatures of 40 °C in a saline environment.

B. Ultrastructural characterization of 980625-4A

Nannochloris eucaryotum has a smooth cell wall documented by SEM micrographs (Wilhelm et al. 1982), as does 980625-4A (Fig. 5). In contrast, the outer cell wall (documented by SEM) of the marine eustigmatophyte previously identified as *Chlorella minutissima* has net-like raised areas (Gladu et al. 1995).

The internal organization of 980625-4A (Fig. 11) is similar to *N. eucaryotum* (Wilhelm et al. 1982). Both contain a single cup-shaped chloroplast, one mitochondrion and one nucleus. TEM micrographs of both algae show a cup-shaped chloroplast with the nucleus and or mitochondrion located near the inside curve of the chloroplast. Both algae have the five-banded thylakoid typical of green algae (Wilhelm et al. 1982). *Nannochloris coccoides* strain SAG 251-1 (originally identified as *Choricystis minor*) and *C. minor* strains KR 1986/11 and 1986/27 are freshwater species (Krienitz et al. 1996) and have similar ultrastructure and organization to 980625-4A as documented by TEM micrographs. Both *C. minor* strains have one nucleus, one mitochondrion, one chloroplast, as does 980625-4A and the mitochondrion and nucleus are located near the inner curve of the cup-shaped chloroplast.

Pyrenoids have not been observed in TEM micrographs of either 980625-4A or *N. eucaryotum*. The presence of a pyrenoid is not a defining characteristic of the genus.

Though pyrenoids are absent in *N. bacillaris* and *N. maculatus*, pyrenoids have been observed in *N. atomus* (Brown and Elfman 1983). Every class of eukaryotic algae have members which posses pyrenoids and members which do not have pyrenoids. For instance, not all members of the genus *Chlorella* contain pyrenoids (Lee 1992, Prescott 1982). The presence or absence of a pyrenoid is not a useful characteristic for distinguishing whether 980625-4A is a member of *Nannochloris*, *Chlorella*, or another genus.

The ultrastructure of 980625-4A is different from the marine eustigmatophytes *Nannochloris oculata* and *Monallantus salina* Bourrelly (Antia et al. 1975) and *Nannochloropsis* sp. (Sukenik et al. 1998). The eustigmatophytes contain several mitochondria and *N. oculata* and *M. salina* contain pyrenoids, whereas 980625-4A contains one mitochondrion and lacks a pyrenoid. The mitochondrion of 980625-4A is larger and is located near the inside curve of the chloroplast, whereas the mitochondria of the eustigmatophytes are smaller and interspersed within the protoplast.

Cell size has been used to distinguish species of *Chlorella* from *Nannochloris*. Descriptions of *Nannochloris* report cell diameters ranging from 0.8 to 3 μm, which would place it among the picoplankton (Krienitz et al. 1996). Most *Chlorella* species are larger nanoplankton (> 3 μm), but there are several small *Chlorella* species. Marine *Chlorella nana* Andreoli et al. one of the smaller *Chlorella* spp., is 1.5-3 μm in diameter (Andreoli et al. 1978). The size of 980625-4A falls within the size range of *C. nana*. In contrast, most documented species of *Chlorella* are larger than 980625-4A: *C. salina* Teodoresco 4-7 μm, *C. ovalis* 3-5 μm x 5-10 μm, *C. marina* 4-6 μm x 7-10 μm and *C. stigmatophora* 4-6 μm (Andreoli et al. 1978).

The TEM micrographs of *Chlorella nana* reveal a cup-shaped chloroplast with starch grains, one mitochondrion and a large vacuole. The mitochondrion is located near the chloroplast, and may be lengthened or sometimes lobed (Rascio et al. 1979). The mitochrondrion of 980625-4A appears bean-shaped in TEM micrographs. The chloroplast of *C. nana* contains two large starch grains filling almost 50 % of the chloroplast volume. The chloroplast of 980625-4A, in all treatments and all micrographs, shows several smaller oval starch grains.

980625-4A shares similar ultrastructural traits with *Nannochloris* spp. and some *Chlorella* spp. The ultrastructure and organization of eustigmatophytes are different from 980625-4A, and assignment of 980625-4A can be excluded from the Eustigmatophyceae. Ultrastructural features alone do not allow assignment of 980625-4A to a specific genus, as it shares ultrastructural traits with several *Nannochloris* and *Chlorella* spp. Various species of both *Chlorella* and *Nannochloris* have been assigned to the classes Trebouxiophyceae and Chlorophyceae, but these assignments are matters of disagreement that can only be resolved with additional genetic and biochemical information.

C. Systematics of 980625-4A based upon ruthenium red staining

Ruthenium red adheres to the acidic polymers (pectin) of the cell wall of some species of algae and has been used as a taxonomic marker in the genus *Chlorella* (Tschermak-Woess 1999, Puncocharova 1994, Takeda 1993a, Krienitz et al. 1999). Ruthenium red treated cells are usually viewed under a light microscope to determine if the cell wall was stained or not. Owing to the small cell size of 980625-4A, light microscopy was unable to provide adequate resolution or contrast to judge the extent of staining. Instead, staining was assessed by centrifuging cell suspensions after addition of

ruthenium red, and the amount of stain remaining in the supernatant was observed. Ruthenium red did not stain the cell walls of 980625-4A, *Nannochloris* spp. UTEX 2378 and UTEX 2379 because the supernatant of the ruthenium red solution was the same color as the original ruthenium red solution. Ruthenium red treatment was determined to be positive for staining of *C. vulgaris, C. saccharophila* and *C. sorokiniana* because the supernatant of the ruthenium red treatment was much lighter than the original ruthenium red solution. These results suggest 980625-4A is not a member of the genus *Chlorella*.

There are several ruthenium red studies with *Nannochloris*, *Chlorella* and closely related algae. The cell wall of *Nannochloris eucaryota* (note spelling) stains with ruthenium red (Tschermak-Woess 1999). *Chlorella* spp. were intensely stained with ruthenium red and different species and different strains within those species reacted differently to staining (Takeda 1991, 1993a, 1993b and 1995; Puncocharova 1994). Five strains of *C. saccharophila* var. *saccharophila* were not stained with ruthenium red (Takeda 1991). In another study, three out of nine strains identified as *C. saccharophila* did stain with ruthenium red (Takeda 1993b). It is impossible to classify algal species solely upon a ruthenium red test, but these results provide important indications of similarities in cell wall composition.

D. Systematics of 980625-4A based upon pigment analysis

Pigment analysis of *Nannochloris* sp. UTEX 2378, *Nannochloris* sp. UTEX 2379, *Chlorella vulgaris, C. saccharophila* and *C. sorokiniana* show similarities and differences when compared to 980625-4A. Among these algae, *Nannochloris* sp. UTEX 2379 stands out as the only alga containing Chl *a* but no Chl *b* or other chlorophylls,

indicating that it does not belong to the genus *Nannochloris*, Class Chlorophyceae. These results suggest UTEX 2379 should be transferred to the Class Eustigmatophyceae, and this conclusion is supported by 18S rDNA sequence analysis (J. A. and M. A. Buchheim personal communication). *Nannochloris oculata* was originally placed into the genus *Nannochloris* but later was found to contain only Chl *a* and was reclassified as *Nannochloropsis* and transferred to the Eustigmatophyceae (Antia et al. 1975).

Nannochloris sp. UTEX 2378 and 980625-4A showed the greatest similarities in carotenoid profiles among all the algae studied (Figs. 27, 28). The major carotenoids of *Nannochloris* sp. UTEX 2378, *Nannochloris* sp. UTEX 2379 and 980625-4A are lutein (labeled C7) and β-carotene (labeled C11). Relative retention times and UV-visible spectra of all eleven carotenoids from 980625-4A match the carotenoids from *Nannochloris* sp. UTEX 2378, but not *Nannochloris* sp. UTEX 2379 (Fig. 27) further evidence that the latter strain is misclassified. Only three carotenoids from 980625-4A, violaxanthin (C3), and two unidentified carotenoids (C4 and C6) are found in *Nannochloris* sp. UTEX 2379. Neither of the two unidentified carotenoids have UV-visible spectra that match vaucheriaxanthin or any known esters. Eustigmatophytes have been reported to usually contain only the xanthophyll pigments violaxanthin and/or vaucheriaxanthin (Gladu et al. 1995). An earlier report by Wilhelm et al. (1982) identified Chl *a* and *b*, β-carotein, lutein, zeaxanthin and neoxanthin *in Nannochloris eucaryotum*. All of these pigments except zeaxanthin were present in 980625-4A. Zeaxanthin is produced in the xanthophyll cycle when algal cells are grown under high light (Hall and Rao 1995). 980625-4A cells were grown under low light and therefore would most likely have undetectable concentrations of zeaxathin.

All three *Chlorella* spp. contain both Chl *a* and *b* as well lutein as their major carotenoid, as does 980625-4A (Fig. 28). *C. vulgaris* and *C. saccharophila* additionally contain violaxanthin as a major carotenoid whereas *C. sorokiniana* and 980625-4A do not. All six algae contain β-carotene, and *C. vulgaris* and *C. saccharophila* have higher relative concentrations than the other four algae. Peak S1 in *C. saccharophila* appears to be the same as C1 from 980625-4A, an unknown carotenoid, and nearly all the carotenoids in *C. saccharophila* match relative retention times and UV-visible spectra with corresponding peaks in 980625-4A. Peak X2 in *C. sorokiniana* is the same as C6 from 980625-4A, an unknown carotenoid.

In summary, the pigments of 980625-4A show similarities to other *Nannochloris* and *Chlorella* species, but the greatest similarity among the algae investigated is to *Nannochloris* sp. UTEX 2378. *Nannochloris* sp. UTEX 2379 shows least similarity to 980625-4A. Based upon the ultrastructure, cell wall staining, and pigment composition, 980625-4A shows greater similarity to the genus *Nannochloris* than to *Chlorella*, but this information alone is not sufficient to establish phylogenetic relationships between 980625-4A and other algae.

E. Systematics of 980625-4A based upon modes of cell propagation

The mode of cell propagation is used as a determining factor in algal taxonomy and has been documented by TEM micrographs in several studies of *Nannochloris* (Wilhelm et al. 1982, Menzel and Wild 1989, Krienitz et al. 1996). Two types of cell propagation are reported to distinguish the genus *Nannochloris* from the genus *Chlorella*. Algae of the genus *Chlorella* are reported to reproduce by autosporulation, in which the

mother cell divides into two or more identical cells and these daughter cells (autospores) form their own distinct cell walls inside of and separate from the mother cell wall. The mother cell wall degenerates or breaks open only after daughter cell cytokinesis is complete. In the classical definition of the genus, *Nannochloris* reproduces by binary fission in which the mother cell wall divides the daughter cells; it also refers to the process of cell division in organisms not possessing a true cellulose cell wall, e. g. *Paramecium* sp., *Dunaliella* sp., and bacteria.

In the original description of the genus *Nannochloris*, the mode of reproduction distinguished *Nannochloris* from *Chlorella* (Naumann 1919). Since then, algae that divide by autosporulation have been assigned to *Nannochloris* even though such assignments conflict with the original genus description. However, Naumann's descriptions of *Nannochloris* did not have the benefit of electron microscopic examination. Though he did not observe remnants of a mother cell wall, it is conceivable that the remnants dissolved too quickly by the autospores to be observed. More recent electron microscopic studies of algae identified as *Nannochloris* primarily documented division producing only two autospores, but some have reported three on some occasions (two small, one large) and, less frequently, four autospores produced from one mother cell (Wilhelm et al. 1982, Menzel and Wild 1989, Tschermak-Woess 1999).

TEM micrographs of 980625-4A were generated from cells grown at 25 and 40 ^{o}C in AS100 at 0, 40, 80, and 120 g-L^{-1} NaCl. In every case, (31), where reproduction was observed, each mother cell produced only two autospores which had cell walls distinct from the mother cell wall (Figs. 14, 15). No evidence of reproduction was observed for the culture grown at 40 ^{o}C and 0 g-L^{-1} salinity. In numerous light

microscopic inspections of 980625-4A from numerous cultures only single cells and double cells were observed, therefore no evidence indicated reproduction formed more than two autospores. In contrast, the genus *Chlorella*, as characterized by Beijerinck in 1890, reproduces by production of 4 or 8 daughter cells from one mother cell (Prescott 1982).

F. Conclusions regarding the systematic assignment of 980625-4A

980625-4A is a poikilotroph, halotolerant, unicellular, coccoid, green alga isolated from an ephemeral saline pond at the SPNWR in Oklahoma. Ultrastructure, pigment composition, ruthenium red staining of the cell wall, mode of reproduction, and the small cell size suggest 980625-4A bears more similarity to the genus *Nannochloris* than to *Chlorella*. Based on 18S rDNA analysis, the alga is most closely related to members of the genus *Nannochloris* Naumann (J. A. and M. A. Buchheim personal communication). The observation of autosporulation in 980625-4A should not prevent its classification into the genus *Nannochloris*. Naumann concluded that formation of only two daughter cells from each mother cell was a characteristic of *Nannochloris* and not *Chlorella*. Naumann was unable to see evidence of the mother cell wall using light microscopy, therefore he assumed binary fission was the mode of cell division. The important distinction of Naumann is not whether cell division involves binary fission or autosporulation into two daughter cells, but rather that two, and only two daughter cells are produced from one mother cell.

I conclude that the genus *Nannochloris* should be characterized by the dominant mode of reproduction yielding two daughter cells per mother cell, regardless of whether

binary fission or autosporulation is involved. The occasional formation of three or four autospores per mother cell does not contradict such characterizations because autospore release may be subject to environmental conditions that alter rates of cell division and mother cell wall degradation. Based on this criterion and the morphological, biochemical, and genetic characteristics described above, the alga 980625-4A should be classified within the genus *Nannochloris*. If 980625-4A is indeed an undescribed species, I propose that the name *Nannochloris oklahomensis* be used.

G. Cell size measured at different temperatures and salinities

Fixation and sample preparation for SEM and TEM cause cellular distortions and can introduce bias that makes quantitative conclusions difficult. When measuring cell size, SEM micrographs are more reliable than TEM micrographs because one cannot be certain that sectioning yields micrographs representative of a cross-section through the center of the cell. Cells fixed for TEM may have different degrees of distortions in different cells even when the same protocol is followed. In some of the 980625-4A TEM micrographs the protoplast pulled away from the cell wall. In a few micrographs the cell wall became thicker and lighter in density losing its sharp definition found in the majority of the cell walls. In micrographs of cells grown at higher salinities, the cell walls were wavy and not a smooth line as in the lower salinities. These cell wall distortions make measuring inaccurate.

The cell size of 980625-4A grown in AS100, 0 g-L^{-1} NaCl is approximately 2.1 ± SD μm, documented by SEM, (n = 7). The mean size of the cells grown under the same conditions and measured from digitally analyzed light microscope photographs was 1.49

± SD μm, (n = 109). The discrepancy in cell size measurements arises because of the tiny size of the cells and the inability of the light microscope to produce images with adequate resolution and contrast. These measurements should not be used to accurately determine the cell size but the comparisons in cell size can be considered reliable because all cells were measured in the same manner.

Light microscope-based measurements of major and minor axes of 980625-4A cells grown at 25 and 40 °C in different salinities show slight (about 6 % variation in mean values at 25 °C and 27 % variation at 40 °C) but statistically significant ($P < 0.01$, 2-Way ANOVA) dependence on salinity, temperature, and salinity-temperature interaction. Cell sizes were smallest for 40 °C and 120 g-L^{-1} salinity. Even though growth rates were approximately zero at 40 °C and 0 g-L^{-1} salinity, light microscopic examination showed no evidence of altered cell size or shape, and it is concluded that cells remained in a lag phase even after a total of 10 days acclimation to the temperature and salinity culture conditions.

The ratio of the major:minor diameters of 980625-4A cells grown at 25 and 40 °C decreased with increasing salinity (2-Way ANOVA, $P < 0.001$) but showed no statistically significant relationship with temperature and salinity-temperature interactions (2-Way ANOVA, $P = 0.89$ and 0.08 respectively). The ratio of the major:minor diameters were normally distrubuted ($P < 0.001$) and may indicate different cell ages in the culture. A linear regression of the ratio of the major:minor diameters of 980625-4A cells grown at 25 and 40 °C and grouped by salinity showed a statistically significant relationship with salinity (slope = –0.00317, $P = 0.001$). The impact of salinity, temperature and salinity-temperature interactions on ratio of the major:minor diameters

of 980625-4A cells may be statistically significant, but the small differences may not be biologically sense significant.

H. Ultrastructure as a function of salinity and temperature

One TEM micrograph from each of the 980625-4A cultures: AS100 at 0, 40, 80, 120 g-L^{-1} NaCl at 25 °C and 40 °C was visually analyzed for salinity- and temperature-dependent ultrastructure changes (Figs. 13, 17-19). The ultrastructure of the cells grown at 40 °C were different from the cells grown at 25 °C. It is not possible to rule out the possibility that the distorted cells in the TEM micrographs for cells grown at 40 °C arise from fixation artifacts. However, these distortions were consistently observed for all cells grown at 40 °C regardless of culture salinity, whereas similar distortions were not observed for any cells grown at 25 °C. Growth rates indicate impaired growth at 40 °C at all salinities relative to cells grown at 25 °C, and culture conditions that gave highest growth at 40 °C (40 and 80 g-L^{-1} salinity) showed the least distorted ultrastructure. Some of the observed ultrastructural changes may result from impaired physiological functioning such as denaturation of cytoskeletal proteins or enzymes and loss of membrane integrity in 980625-4A at 40 °C.

I. Osmolyte analyses for two salinities

The HPLC-mass spectrometric (HPLC-MS) analysis of perchloric acid extracts provides a sensitive method for identifying an assortment of cellular solutes including amino acids, mono- and di-saccharides, polyols such as glycerol, and other water-soluble substances such as glycine betaine and ectoine. Identifications are based upon molecular

masses of ionized molecules formed by attachment of either H^+ or NH_4^+ which are present in the HPLC mobile phase. Ionization is further enhanced by use of a corona discharge from a needle held at 5500 volts. The HPLC separation used in this study was based on a strong cation-exchange column. As a result, neutral molecules such as glycerol and glucose are not strongly retained and elute quickly, whereas amino acids that have a positive charge at the mobile phase pH elute from the column later. To my knowledge, this is the first application of ion-exchange HPLC coupled to mass spectrometry for identification of osmolytes.

The most abundant substances observed in perchloric acid extracts of 980625-4A were proline, glycerol and glucose and a lesser amount of glucosylglycerol (Figs. 29, 30). The high concentrations of these compounds relative to amino acids and other secondary metabolites suggest their concentrations are sufficient for them to be considered osmolytes. All three of the most abundant osmolytes (proline, glycerol, and glucose) were higher in abundance in the cells grown at 40 $g\text{-}L^{-1}$ salinity relative to cells grown at 0 $g\text{-}L^{-1}$ salinity, with the percentage increase being by far greatest for proline (Table 4).

Proline, betaine, ectoines, and N-acetylated diamino acids are the major osmolytes in nature (Galinski et al. 1997). The amino acid proline is one of the major osmolytes in many photosynthetic organisms including *Nannochloris bacillaris*, *Synechococcus* Nageli 1849 spp. PCC7942 and PCC7418, *Synechocystis* Sauvageau 1892 sp. PCC6803, the Antarctic sea-ice diatoms *Chaetoceros* Ehrenberg 1844 sp., *Navicula* Bory 1822 sp., polar macroalgae, and higher plants (Brown 1982a, Nothnagel 1995, Kueck 1997, Strizhov et al. 1997, Yoshiba et al. 1997, Fulda et al. 1999). Proline

organizes water molecules by forming hydrogen bonds to as many as three to five per proline molecule and reduces the water potential (Galinski et al. 1997).

Glycerol is the major osmolyte in *Dunaliella* and *Chlamydomonas* (Hellebust 1976, Pick 1998). Glucosylglycerol is used as an osmolyte by euryhaline isolates from marine and freshwater habitats and the cyanobacteria *Microcoleus chthonoplastes*, *Synechococcus* Sauvageau 1892 *sp.*, *Dermocarpa* Crouan 1858, and *Myxosarcina* Printz 1921 (Mackay et al. 1984, Reed 1984, Karsten 1996, Warr et al. 1985).

The most extensive investigations of osmolytes in halotolerant eukaryotic algae have focused on accumulation of glycerol by species of *Dunaliella*.

J. Growth rates as a function of salinity and temperature

The experimental temperature of 40 °C was chosen as the upper limit for 980625-4A experiments because range finding experiments suggested 40 °C is close to the alga's upper growth temperature at 50 g-L^{-1} salinity. Forty °C is similar to maximum water temperature recorded in the summer months at the SPNWR. However, 980625-4A cultured at 40 °C in AS100 at 0 g-L^{-1} NaCl did not grow but 980625-4A grew at rates of 0.20 to 0.32 d^{-1} at other salinities at this temperature (Fig. 26). Growth rates at 25 °C ranged from 0.48 to 0.67 d^{-1} over the range of salinities (0-120 g-L^{-1}). This finding suggests that osmolytes induced by elevated salinities and heat shock proteins produced at higher temperatures may play protective roles (Adams 1991, Brown 1982a) because 980625-4A cells grew well at 40 °C in higher salinities. Absolute growth rates for 980625-4A were similar in magnitude to those reported for *Nannochloris bacillaris* at 20 °C (Brown 1982a), and somewhat less than growth rates reported for *Dunaliella bardawil*

and *D. salina* over all salinities from 58-230 g-L^{-1} (Ben-Amotz and Avron 1979). Growth of *D. tertiolecta* exceeded 0.7 d^{-1} at NaCl concentrations ranging from 0.7 to 82 g-L^{-1} but dropped sharply at salinities less than 0.7 and above 105 g-L^{-1} (McLachlan 1960). In contrast to 980625-4A, which showed only a slight relationship between salinity and growth over 0-120 g-L^{-1} at 25 °C, growth of *N. bacillaris* reached maxima at low salinities (about 0.8 d^{-1} for 0-10 g-L^{-1} salinity) and decreased monotonically to a minimum of about 0.2 d^{-1} at 105 g-L^{-1} salinity. These results suggest growth of 980625-4A is less salinity-dependent than *N. bacillaris*, but interpretation of comparisons of growth rates measured by different laboratories must be tempered by recognition that growth rates are influenced by culture conditions.

The temperature growth range for *Chlorococcum littorale*, a marine coccoid green alga, is 15-28 °C and, unlike 980625-4A, the growth rate showed strong temperature dependence. *C. littorale* can not survive temperatures 30 °C or higher, (Chihara et al. 1994), unlike 980625-4A, which can grow at 40 °C and survives 45 °C for at least 2 hours (Henley et al., in prep). The growth rates of the fresh water diatom *Asterionella* Hassall 1850 and dinoflagellate *Peridinium cinctum fa.* westii (Lemm.) Lef are temperature dependent (Talling 1955, Lindstrom 1984). The minor effects of temperature on the growth rate of 980625-4A are rare over this temperature range, suggesting 980625-4A has unusual thermotolerance characteristics.

The range of environments, such as salinities and temperatures, in which algae can live has been used to aid classification (Talling 1955, Ostroff et al. 1980). The growth rate of 980625-4A cultures grown at 25 °C in AS100 at 0, 40, 80, 120 g-L^{-1} NaCl decreased from 40 to 120 g-L^{-1} NaCl. Cells growing in elevated salinity allocate energy

towards the production of osmolytes and salt induced proteins and as a consequence may display a reduced growth rate (Ha and Thompson 1992, Shan et al. 1996, Chitlaru et al. 1997, Fisher et al. 1997). Most halotolerant microalgae have higher growth rates at lower salinities (Brown 1982a). *Chlorococcum littorale,* a marine coccoid green alga, exhibits a maximum growth rate at 15 $g\text{-}L^{-1}$ NaCl and about half that rate at 90 $g\text{-}L^{-1}$ NaCl (Chihara et al. 1994). *Dunaliella salina*, a well-studied halophilic alga, exhibited the highest growth rate when grown at 26 $g\text{-}L^{-1}$ NaCl, and decreased at concentrations of 100 to 300 $g\text{-}L^{-1}$ NaCl (Al-Hasan et al. 1987). *Nannochloris bacillaris*, isolated from the Indian Ocean, grew at 0.8 d^{-1} at 9 $g\text{-}L^{-1}$ NaCl, but only, 0.25 d^{-1} at 105 $g\text{-}L^{-1}$ NaCl (Brown 1982a). *Dunaliella bardawil* grew fastest (2.4 d^{-1}) at 58 g $\text{-}L^{-1}$ NaCl and decreased with increased salinity to 1.4 d^{-1} at 234 $g\text{-}L^{-1}$ NaCl. *Dunaliella salina* grew fastest (3.4 d^{-1}) at 58 $g\text{-}L^{-1}$ NaCl and slowest (1.8 d^{-1}) when grown in 234 $g\text{-}L^{-1}$ NaCl (Ben-Amotz and Avron 1979). 980625-4A grew fastest (0.67 d^{-1}) when grown at 25 °C in 40 $g\text{-}L^{-1}$ NaCl and the slowest (0.19 d^{-1}) at 40 °C in 120 $g\text{-}L^{-1}$ NaCl (Fig. 26).

The cell densities of unicellular coccoid algae are easily measured and are used to assess the acclimation to stresses. The change in cell density of 980625-4A was measured during the early to late logarithmic growth phase for cultures grown at 25 and 40 °C in AS100 at 0, 40, 80, 120 $g\text{-}L^{-1}$ NaCl. The cultures did not grow measurably at 40 °C, 0 $g\text{-}L^{-1}$ NaCl. The logarithmic growth phase for 25 and 40 °C was about nine and six days respectively. The treatments with the highest change in cell density were the lower salinity cultures 25 °C, 0 and 40 $g\text{-}L^{-1}$ NaCl and 40 °C, 40 $g\text{-}L^{-1}$ NaCl and the lowest change was at 25 °C, 80 $g\text{-}L^{-1}$ NaCl. The change in cell density decreased dramatically at both temperatures at 80 and 120 $g\text{-}L^{-1}$ NaCl (Figs. 24, 25). In comparison, the maximum

cell density in *Dunaliella bardawil* and *D. salina* was the highest when grown in 58 $g\text{-}L^{-1}$ NaCl and lowest in 292 $g\text{-}L^{-1}$ NaCl (Ben-Amotz and Avron 1979).

K. Conclusions about responses to salinity and temperature stress

Several conclusions may be drawn from the responses of 980625-4A to elevated salinities and temperatures. The response of this organism to higher salinity was to produce higher concentrations of osmolytes. Cultures grown at 25 °C and 40 $g\text{-}L^{-1}$ salinity produced higher concentrations of three abundant osmolytes than at 0 $g\text{-}L^{-1}$ salinity. Osmolytes can play two roles important in poikilotrophs. First, the osmolyte serves to maintain osmotic balance between compartments by increasing the water potential. The second involves a role as a "compensatory solute" which stabilizes the hydration shell surrounding proteins thus protecting the protein from denaturation from ions or other stresses (Clark 1985). Compensatory solutes appear to function by stabilizing protein folding. Both proline and glycerol have been recognized as effective compensatory solutes capable of preserving macromolecular function even in the presence of otherwise deleterious concentrations of salts such as NaCl (Borowitzka and Brown 1974, Brown 1990, Clark 1985, Gilles 1997). Proline also can stabilize proteins against thermal denaturation (Arakawa and Timasheff 1985). Osmolyte production is expected to stabilize proteins to both hypersaline environments and elevated temperatures; this is supported by the nonexistent growth of 4A at 40 °C and 0 $g\text{-}L^{-1}$ NaCl, conditions where osmolyte concentrations are expected to be at a minimum.

The ability to produce multiple osmolytes allows multiple mechanisms that can regulate algal acclimation to osmotic and thermal stresses. The unusual tolerance of

980625-4A to elevated salinities and temperatures suggests a great ability to acclimate compared to other algae. Though the production of multiple osmolytes has been observed for several algae (Kirst 1989), many studies have focused on the dominant osmolyte (e.g. glycerol in *Dunaliella*), and the extent to which multiple osmolytes are produced as a response to stressful environments has yet to be fully established.

The dominant osmolyte produced by 980625-4A is proline which is a nitrogen-containing solute. Whether proline can be biosynthesized in quantities sufficient to serve this role under nitrogen-deficient conditions is unclear. The ability of 980625-4A to withstand similar ranges of salinity and temperature in field conditions may be influenced by the ability of 980625-4A to produce sufficient levels of osmolytes; such factors may limit the occurrence of this alga in aquatic ecosystems.

980625-4A did not grow at 40 °C in AS100 at 0 $g\text{-}L^{-1}$ NaCl, but the salinity-independent growth rates at the other experimental temperatures and salinities suggest osmolytes and or some other factor(s) protect the alga from the stresses of temperature.

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APPENDIXES

Appendix 1. Raw data for 980625-4A cells grown at 25C in different salinities. The values are the measurements of absorbance at 750 nm.

Day	1	2	3	4	5	6	7	8	9	10	11
0 g/L NaCl											
Flask 1	0.009	0.009	0.013	0.028	0.039	0.059	0.072	0.077	0.960	0.114	0.144
Flask 2	0.009	0.009	0.013	0.023	0.038	0.043	0.076	0.077	0.101	0.114	0.145
Flask 3	0.011	0.009	0.013	0.025	0.039	0.052	0.067	0.078	0.098	0.114	0.148
Flask 4	0.010	0.010	0.015	0.026	0.034	0.055	0.077	0.086	0.105	0.115	0.145
40 g/L NaCl											
Flask 1	0.010	0.007	0.013	0.017	0.028	0.039	0.057	0.066	0.095	0.109	0.140
Flask 2	0.008	0.007	0.013	0.020	0.030	0.044	0.062	0.075	0.010	0.118	0.152
Flask 3	0.010	0.007	0.014	0.020	0.029	0.045	0.061	0.076	0.101	0.113	0.150
Flask 4	0.008	0.007	0.012	0.019	0.026	0.044	0.062	0.061	0.088	0.100	0.129
80 g/L NaCl											
Flask 1	0.011	0.005	0.010	0.015	0.015	0.016	0.027	0.030	0.041	0.046	0.063
Flask 2	0.011	0.007	0.010	0.015	0.013	0.022	0.032	0.030	0.043	0.046	0.064
Flask 3	0.011	0.008	0.010	0.015	0.012	0.018	0.026	0.030	0.044	0.047	0.065
Flask 4	0.011	0.008	0.010	0.015	0.013	0.020	0.030	0.023	0.043	0.047	0.062
120 g/L NaCl											
Flask 1	0.009	0.005	0.007	0.010	0.006	0.006	0.009	0.011	0.018	0.014	0.022
Flask 2	0.008	0.005	0.008	0.010	0.006	0.010	0.016	0.009	0.014	0.011	0.025
Flask 3	0.006	0.005	0.008	0.010	0.009	0.011	0.018	0.010	0.023	0.016	0.027
Flask 4	0.005	0.005	0.007	0.010	0.009	0.012	0.017	0.007	0.019	0.016	0.022

Appendix 2. Raw data for 980625-4A cells grown at 40C in different salinities. The values are the measurements of absorbance at 750 nm.

Day	1	2	3	4	5	6
0 g/L NaCl						
Flask 1	0.040	0.040	0.035	0.036	0.042	0.042
Flask 2	0.039	0.037	0.039	0.041	0.042	0.038
Flask 3	0.039	0.043	0.034	0.040	0.038	0.038
Flask 4	0.037	0.040	0.033	0.040	0.038	0.038
40 g/L NaCl						
Flask 1	0.038	0.054	0.068	0.090	0.126	0.180
Flask 2	0.035	0.050	0.063	0.084	0.125	0.183
Flask 3	0.035	0.049	0.063	0.084	0.119	0.165
Flask 4	0.035	0.049	0.062	0.088	0.118	0.165
80 g/L NaCl						
Flask 1	0.026	0.038	0.046	0.065	0.084	0.102
Flask 2	0.035	0.041	0.049	0.065	0.085	0.105
Flask 3	0.027	0.040	0.049	0.063	0.086	0.101
Flask 4	0.035	0.039	0.045	0.065	0.085	0.101
120 g/L NaCl						
Flask 1	0.026	0.032	0.034	0.043	0.053	0.061
Flask 2	0.024	0.029	0.030	0.039	0.048	0.056
Flask 3	0.026	0.033	0.035	0.045	0.053	0.059
Flask 4	0.024	0.029	0.030	0.036	0.051	0.059

Appendix 3. Raw data for 980625-4A cells grown at 25C in 0 g/L NaCl.
Measurements are in microns.

Cell Number	Major Axis	Minor Axis	Maj/Mi	Average Major	Standard Dev Major	Average Minor	Standard Dev Minor	Average Major:Minor	Standard Dev Major:Minor
1	1.327	1.152	1.152	1.469	0.109	1.276	0.100	1.154	0.087
2	1.310	1.136	1.154						
3	1.602	1.289	1.242						
4	1.507	1.288	1.170						
5	1.413	1.204	1.174						
6	1.167	1.080	1.081						
7	1.469	1.355	1.084						
8	1.488	1.331	1.118						
9	1.482	1.465	1.012						
10	1.450	1.295	1.120						
11	1.698	1.519	1.118						
12	1.501	1.252	1.200						
13	1.347	1.207	1.116						
14	1.482	1.210	1.225						
15	1.465	1.323	1.108						
16	1.415	1.181	1.198						
17	1.482	1.286	1.152						
18	1.436	1.160	1.238						
19	1.398	1.305	1.072						
20	1.432	1.287	1.113						
21	1.470	1.336	1.100						
22	1.498	1.153	1.299						
23	1.615	1.219	1.324						
25	1.417	1.258	1.127						
26	1.445	1.275	1.134						
27	1.717	1.215	1.413						
28	1.521	1.390	1.094						
29	1.451	1.339	1.084						
30	1.447	1.418	1.020						
31	1.602	1.353	1.184						

Cell Number	Major Axis	Minor Axis	Maj/Min	Cell Number	Major Axis	Minor Axis	Maj/Min
32	1.821	1.510	1.206	67	1.589	1.206	1.318
33	2.004	1.347	1.487	68	1.288	1.122	1.148
34	1.789	1.287	1.389	69	1.542	1.111	1.388
35	1.330	1 171	1.135	70	1.218	0.848	1.437
37	1.798	1.544	1.164	71	1.330	1.249	1.064
38	1.584	1.165	1.359	72	1.532	1.273	1.203
40	1.547	1.460	1.059	73	1.305	1.054	1.238
41	1.374	1.103	1.246	74	1.436	1.021	1.406
42	1.744	1.427	1 222	75	1.239	1.100	1.127
43	1.507	1.072	1.406	76	1.444	1.169	1.235
44	1.629	1.615	1.009	77	1.433	1.039	1.380
45	1.440	1.193	1.207	78	1.673	1.304	1.283
46	1.514	1.278	1.184	79	1.406	1.094	1.285
47	1.414	1.249	1.132	80	1.397	1.267	1.102
48	1.498	1.343	1.115	81	1.850	1.600	1.157
49	0.749	0.522	1.435	82	1.622	1.249	1.299
50	1.197	1.014	1.180	83	1.301	1.180	1.103
51	1.424	1.125	1.266	84	1.392	1.351	1.031
52	1.403	1.348	1.041	85	1.321	0.944	1.400
53	1.503	1.338	1.124	86	1.490	1.081	1.378
54	1.458	1 134	1.286	87	1.383	1.161	1.191
55	1.327	1 113	1.192	88	1.485	1.414	1.051
56	1.357	1.096	1.238	89	1.434	1.058	1.356
57	1.358	1.115	1.218	90	1.478	1.207	1.224
58	1.213	1 134	1.070	91	1.628	1.423	1.144
59	1.506	1.267	1.188	92	1.577	1.195	1.319
60	1.059	1.006	1.053	93	1.400	1.115	1.255
61	1.289	0.866	1.490	94	1.462	1.344	1.088
62	1.400	1.081	1.294	95	1.392	1.089	1.279
63	1.542	1.283	1.202	96	1.446	0.980	1.475
64	1.592	1.574	1.012	97	1.645	1.300	1.265
65	1.418	1 145	1.238	98	1.583	1.261	1.256
66	1.621	1.445	1.121	99	1.985	1.852	1.071

Cell Number	Major Axis	Minor Axis	Maj/Min
100	1.525	1.442	1.058
101	1.559	1.252	1.246
102	1.731	1.379	1.256
103	1.534	1.336	1.148
104	1.581	1.210	1.307
105	1.287	1.035	1.244
106	1.786	1.561	1.144
107	1.689	1.597	1.058
108	1.578	1.310	1.205
109	1.553	1.388	1.119
110	1.948	1.839	1.059
111	1.682	1.567	1.073
112	1.591	1.482	1.074
113	1.682	1.425	1.180

Appendix 4. Raw data for 980625-4A cells grown at 25C in 40 g/L NaCl.
Measurements are in microns.

Cell Number	Major Axis	Minor Axis	Maj / Min	Average Major	Standard Dev Major	Average Minor	Standard Dev Minor	Average Major:Minor	Standard Dev Major:Minor
1	1.501	1.452	1.034	1.556	0.124	1.329	0.118	1.174	0.081
2	1.587	1.464	1.085						
3	1.482	1.325	1.119						
4	1.551	1.219	1.272						
5	1.676	1.526	1.098						
6	1.703	1.404	1.213						
7	1.612	1.230	1.310						
8	1.467	1.313	1.117						
9	1.443	1.178	1.226						
10	1.784	1.435	1.243						
11	1.667	1.587	1.051						
12	1.551	1.385	1.120						
13	1.501	1.246	1.205						
14	1.615	1.445	1.118						
15	1.524	1.387	1.099						
16	1.520	1.287	1.181						
17	1.730	1.412	1.225						
18	1.489	1.328	1.121						
19	1.437	1.323	1.086						
20	1.578	1.265	1.247						
22	1.358	1.166	1.165						
23	1.589	1.209	1.314						
24	1.432	1.240	1.155						
25	1.870	1.398	1.338						
26	1.419	1.172	1.211						
27	1.684	1.506	1 118						
28	1.671	1.440	1 160						
29	1.443	1.164	1.239						
30	1.504	1.253	1.200						
31	1.339	1.253	1.069						
32	1.494	1.195	1.250						

Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min
33	1.528	1.198	1.276	66	1.691	1.543	1.096	99	1.665	1.320	1.261
34	1.516	1.484	1.022	67	1.669	1.498	1.114	100	1.936	1.575	1.229
35	1.655	1.504	1.101	68	1.514	1.346	1.125	101	1.756	1.544	1.138
36	1.499	1.358	1.104	69	1.614	1.377	1.173	102	1.655	1.517	1.091
37	1.739	1.354	1.284	70	1.927	1.582	1.218	103	1.418	1.324	1.070
38	1.311	1.161	1.130	71	1.902	1.673	1.137	104	1.315	1.216	1.081
39	1.821	1.598	1.140	72	1.676	1.405	1.192	105	1.645	1.222	1.347
40	1.384	1.258	1.100	73	1.769	1.393	1.270	106	1.640	1.335	1.228
41	1.327	1.233	1.076	74	1.701	1.389	1.225	107	1.626	1.397	1.164
42	1.759	1.597	1.102	75	1.799	1.545	1.165	108	1.812	1.311	1.382
43	1.747	1.393	1.254	76	1.380	1.263	1.092	109	1.492	1.297	1.151
44	1.527	1.207	1.265	77	1.711	1.556	1.099	110	1.484	1.416	1.048
45	1.473	1.421	1.037	78	1.587	1.538	1.032	111	1.637	1.297	1.262
46	1.627	1.451	1.121	79	1.666	1.431	1.164	112	1.435	1.362	1.053
47	1.642	1.368	1.200	80	1.511	1.229	1.230	113	1.802	1.671	1.079
48	1.416	1.320	1.072	81	1.506	1.238	1.217	114	1.556	1.321	1.178
49	1.708	1.365	1.251	82	1.820	1.229	1.481	115	1.490	1.223	1.218
50	1.519	1.485	1.023	83	1.143	0.948	1.206	116	1.456	1.164	1.251
51	1.623	1.319	1.231	84	1.481	1.434	1.033	117	1.634	1.226	1.333
52	1.554	1.272	1.222	85	1.683	1.521	1.106	118	1.822	1.646	1.107
53	1.555	1.440	1.080	86	1.774	1.506	1.178	119	1.565	1.392	1.124
54	1.516	1.036	1.463	87	1.736	1.493	1.163	120	1.494	1.194	1.251
55	1.436	1.339	1.073	88	1.742	1.552	1.123	121	1.777	1.562	1.137
56	1.758	1.325	1.327	89	1.566	1.449	1.081	122	1.893	1.652	1.146
57	1.467	1.294	1.133	90	1.668	1.409	1.184	123	1.719	1.516	1.134
58	1.830	1.740	1.052	91	1.513	1.291	1.172	124	1.571	1.241	1.266
59	1.618	1.342	1.206	92	1.455	1.196	1.217	125	1.896	1.489	1.273
60	1.564	1.370	1.142	93	1.675	1.332	1.257	126	1.837	1.440	1.276
61	1.647	1.236	1.333	94	1.403	1.152	1.218	127	1.851	1.684	1.099
62	1.502	1.361	1.104	95	1.589	1.345	1.182	128	1.911	1.834	1.042
63	1.787	1.473	1.213	96	1.496	1.282	1.167	129	1.803	1.512	1.192
64	1.967	1.554	1.266	97	1.458	1.244	1.173	130	1.314	1.166	1.127
65	1.809	1.530	1.182	98	1.514	1.215	1.246	131	1.567	1.360	1.152

Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min
132	1.655	1.429	1.157	165	1.218	1.124	1.084	198	1.498	1.316	1.138
133	1.551	1.274	1.217	166	1.488	1.264	1.177	199	1.344	1.088	1.235
134	1.945	1.729	1.125	167	1.464	1.268	1.154	200	1.580	1.355	1.166
135	1.528	1.253	1.220	168	1.404	1.204	1.167	201	1.476	1.348	1.095
136	1.709	1.338	1.277	169	1.748	1.168	1.496	20	1.310	0.936	1.399
137	1.878	1.658	1.132	170	1.204	1.037	1.161	2	1.583	1.352	1.171
138	1.812	1.693	1.071	171	1.505	1.359	1.107	203	1.402	1.088	1.288
139	1.849	1.563	1.183	172	1.375	1.181	1.164	204	1.589	1.458	1.090
140	1.637	1.325	1.235	173	1.157	0.940	1.231	205	1.665	1.387	1.201
141	1.758	1.397	1.258	174	1.539	1.280	1.202	206	1.497	1.334	1.122
142	1.656	1.480	1.119	175	1.362	1.232	1.106	207	1.870	1.590	1.176
143	1.513	1.322	1.145	176	1.422	1.289	1.103	208	1.613	1.250	1.290
144	1.767	1.536	1.151	177	1.251	0.945	1.324	209	1.597	1.073	1.488
145	1.655	1.404	1.179	178	1.351	1.059	1.276	210	1.538	1.262	1.219
146	1.579	1.320	1.196	179	1.440	1.067	1.350	211	1.531	1.190	1.286
147	1.652	1.405	1.176	180	1.394	1.223	1.140	212	1.526	1.190	1.282
148	1.883	1.553	1.213	181	1.336	1.105	1.209	213	1.330	1.093	1.216
149	1.645	1.539	1.068	182	1.518	1.159	1.310	214	1.583	1.313	1.206
150	1.395	1.244	1.121	183	1.508	1.364	1.106	215	1.508	1.378	1.094
151	1.544	1.144	1.350	184	1.662	1.127	1.475	216	1.461	1.277	1.144
152	1.584	1.239	1.278	185	1.500	1.114	1.347	217	1.640	1.224	1.340
153	1.534	1.354	1.133	186	1.306	1.013	1.290	218	1.591	1.261	1.262
154	1.404	1.254	1.120	187	1.578	1.283	1.230	219	1.676	1.342	1.249
155	1.561	1.322	1.180	188	1.295	1.178	1.099	220	1.508	1.324	1.139
156	1.895	1.562	1.213	189	1.422	1.018	1.397	221	1.642	1.403	1.170
157	1.405	1.138	1.235	190	1.251	1.202	1.040	222	1.500	1.212	1.237
158	1.349	1.091	1.237	191	1.485	1.446	1.027	223	1.953	1.629	1.199
159	1.474	1.148	1.284	192	1.506	1.314	1.147	224	1.711	1.475	1.160
160	1.526	1.311	1.163	193	1.432	0.979	1.462	225	1.607	1.482	1.084
161	1.484	1.267	1.171	194	1.459	1.220	1.196	226	1.792	1.551	1.156
162	1.577	1.199	1.315	195	1.288	0.907	1.419	227	1.528	1.325	1.154
163	1.162	0.792	1.466	196	1.461	1.346	1.085	228	1.791	1.637	1.094
164	1.476	1.322	1.117	197	1.489	1.272	1.171	229	1.478	1.213	1.218

Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min
230	1.446	1.196	1.209	263	1.610	1.457	1.105	297	1.320	1.090	1.211
231	1.389	1.270	1.094	264	1.386	1.163	1.191	298	1.535	1.465	1.048
232	1.610	1.343	1.199	265	1.542	1.104	1.397	299	1.851	1.488	1.244
233	1.492	1.262	1.182	267	1.571	1.384	1.135	300	1.462	1.150	1.271
234	1.505	1.445	1.041	268	1.467	1.432	1.025	301	1.070	1.022	1.047
235	1.756	1.465	1.198	269	1.944	1.636	1.188	302	1.270	1.238	1.026
236	1.647	1.376	1.197	270	1.609	1.286	1.251	303	1.520	1.246	1.220
237	1.602	1.245	1.287	271	1.491	1.001	1.490	304	1.363	1.126	1.211
238	1.752	1.413	1.239	272	1.636	1.310	1.249	305	1.469	1.426	1.030
239	1.641	1.527	1.074	273	1.643	1.470	1.118	306	1.482	1.352	1.096
240	1.583	1.331	1.189	274	1.694	1.395	1.214	307	1.353	1.151	1.175
241	1.156	0.821	1.407	275	1.596	1.359	1.174	308	1.441	1.346	1.071
242	1.677	1.583	1.059	276	1.598	1.364	1.171	309	1.202	0.822	1.462
243	1.527	1.114	1.371	277	1.702	1.300	1.310	310	1.079	0.770	1.401
244	1.559	1.252	1.246	278	1.758	1.622	1.084	311	1.422	1.302	1.092
245	1.516	1.395	1.087	279	1.446	1.126	1.284	312	1.325	1.202	1.102
246	1.381	1.080	1.278	281	1.861	1.567	1.187	313	1.304	0.912	1.430
247	1.678	1.408	1.192	281	1.541	1.365	1.129	314	1.358	1.167	1.163
248	1.499	1.371	1.094	282	1.644	1.403	1.172	135	1.465	1.305	1.123
249	1.323	1.109	1.192	283	1.561	1.365	1.143	316	1.449	1.332	1.088
250	1.318	1.189	1.108	284	1.681	1.562	1.076	317	1.566	1.279	1.224
251	1.275	1.233	1.034	285	1.935	1.626	1.190	318	1.629	1.560	1.044
252	1.704	1.548	1.100	286	1.891	1.618	1.169	319	1.754	1.498	1.171
253	1.380	1.276	1.082	287	1.495	1.051	1.423	320	1.438	1.154	1.246
254	1.206	0.855	1.410	288	1.964	1.611	1.219	321	1.445	1.163	1.242
255	1.504	1.251	1.202	289	1.879	1.515	1.240	322	1.284	0.913	1.408
256	1.428	1.318	1.084	290	1.588	1.270	1.250	323	1.554	1.321	1.176
257	1.406	0.980	1.435	291	1.430	1.218	1.175	324	1.313	1.133	1.159
258	1.715	1.340	1.280	292	1.470	1.210	1.215	325	1.394	1.083	1.287
259	1.619	1.439	1.125	293	1.713	1.576	1.087	326	1.634	1.457	1.122
260	1.339	1.250	1.071	294	1.807	1.566	1.154	327	1.559	1.386	1.125
261	1.449	1.235	1.173	295	1.363	1.248	1.092	328	1.212	1.099	1.103
262	1.652	1.524	1.084	296	1.665	1.500	1.110	329	1.594	1.359	1.173

Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min
330	1.471	1.031	1.427	364	1.696	1.358	1.249
331	1.968	1.658	1.187	365	1.706	1.480	1.153
332	1.491	1.225	1.217	366	1.652	1.469	1.125
333	1.587	1.176	1.350	367	1 146	1.063	1.078
334	1.519	1.222	1.243	368	1.590	1.337	1.189
335	1.263	1.061	1 191	369	1.440	1.208	1.192
336	1.655	1.321	1.253	370	1.471	1.410	1.043
337	1.479	1.435	1.031	371	1.874	1.477	1.269
338	1.694	1.407	1.204	372	1.640	1.426	1.150
339	1.424	1.279	1.114	373	1.691	1.587	1.066
340	1.402	1.194	1.174				
341	1.507	1.205	1.250				
342	1.708	1.225	1.395				
343	1.514	1.260	1.201				
345	1.571	1.326	1.184				
346	1.676	1.263	1.326				
347	1.642	1.491	1.101				
348	1.558	1.178	1.323				
349	1.505	1.292	1.165				
350	1.297	1.207	1.074				
351	1.610	1.550	1.039				
352	1.604	1.443	1.112				
353	1.660	1.456	1.140				
354	1.572	1.471	1.069				
355	1.421	1.379	1.031				
356	2.065	1.794	1.151				
357	1.616	1.262	1.280				
358	1.791	1.516	1.181				
359	1.562	1.377	1 134				
360	1.836	1.487	1.235				
361	1.656	1.503	1 102				
362	1.548	1 173	1.320				
363	1.482	1.326	1 118				

Appendix 5. Raw data for 980625-4A cells grown at 25C at 80 g/L NaCl.
Measurements are in microns.

Cell Number	Major Axis	Minor Axis	Maj / Min	Average Major	Standard Dev Major	Average Minor	Standard Dev Minor	Average Major:Minor	Standard Dev Major:Minor
1	1.551	1.262	1.229	1.355	0.133	1.150	0.098	1.181	0.104
2	1.280	1.242	1.031						
3	1.403	1.013	1.385						
4	1.236	1.141	1.083						
5	1.441	1.307	1.103						
6	1.535	1.279	1.200						
7	1.376	0.996	1.382						
8	1.328	1.128	1.177						
9	1.396	1.263	1.105						
10	1.121	1.062	1.055						
11	1.601	1.249	1.283						
12	1.315	1.138	1.155						
13	1.523	1.233	1.235						
14	1.242	1.112	1.117						
15	1.321	1.134	1.164						
16	1.374	1.172	1.172						
17	1.156	1.075	1.076						
18	1.373	1.116	1.230						
19	1.444	1.285	1.123						
20	1.246	0.988	1.261						
21	1.116	0.993	1.123						
22	1.295	1.125	1.151						
23	1.530	1.124	1.361						
24	1.567	1.109	1.413						
25	1.410	1.248	1.130						
26	1.265	1.056	1.198						
27	1.342	1.267	1.059						
28	1.206	1.046	1.153						
29	1.301	1.175	1.108						

Cell Number	Major Axis	Minor Axis	Maj / Mi	Cell Number	Major Axis	Minor Axis	Maj / Mi	Cell Number	Major Axis	Minor Axis	Maj / Min
30	1.733	1.464	1.184	61	1.426	1.152	1.238	92	1.494	1.262	1.184
31	2.005	1.762	1.138	62	1.333	1.140	1.169	93	1.153	0.915	1.260
32	1.200	1.138	1.054	63	1.546	1.333	1.159	94	1.481	1.381	1.073
33	1.558	1.390	1.121	64	1.371	1.133	1.209	95	1.569	1.291	1.215
34	1.431	1.335	1.072	65	1.517	1.335	1.137	96	1.498	1.229	1.219
35	1.694	1.506	1.125	66	1.382	1.049	1.318	97	1.769	1.566	1.130
36	1.369	1.228	1.115	67	1.761	1.574	1 119	98	1.624	1.468	1.107
37	1.749	1.421	1.231	68	1.469	1.384	1.076	99	1.765	1.423	1.240
38	1.834	1.534	1.195	69	1.540	1.333	1 155	100	1.587	1.444	1.099
39	1.662	1.418	1.172	70	1.519	1.171	1.297	101	1.478	1.376	1.074
40	1.707	1.486	1.148	71	1.493	1.059	1.409	102	1.672	1.340	1.248
41	1.448	1.251	1.157	72	1.783	1.513	1.178	103	1.565	1.447	1.081
42	1.364	1.029	1.326	73	1.630	1.389	1.173	104	1.471	1.285	1.145
43	1.296	1.184	1.094	74	1.415	1.344	1.053	105	1.546	1.314	1 177
44	1.295	1.121	1.156	75	1.660	1.559	1.065	106	1.552	1.337	1.161
45	1.403	1.111	1.264	76	1.450	1.102	1.316	107	1.396	1.196	1.167
46	1.315	1.174	1.120	77	1.769	1.289	1.372	108	1.555	1.185	1.313
47	1.335	1.155	1.156	78	1.554	1.245	1.248	109	1.340	1.275	1.051
48	1.654	1.436	1.152	79	1.312	1.038	1.264	110	1 952	1.680	1.162
49	1.443	1.233	1.170	80	1.518	1.195	1.270	112	1.311	1 153	1.137
50	1.265	1.183	1.069	81	1.388	1.291	1.075	113	1.597	1.367	1.169
51	1.399	1.254	1.115	82	1.255	1.181	1.062	114	1.456	1.259	1.156
52	1.232	1.058	1.164	83	1.582	1.296	1.220	115	1.297	1.240	1.047
53	1.477	1.429	1.034	84	1.560	1.344	1.161	116	1.778	1.627	1.093
54	1.233	1.035	1.192	85	1.260	1.063	1.185	117	2.074	1.683	1.232
55	1.460	1.304	1.120	86	1.762	1.543	1.141	118	1.496	1.260	1.187
56	1.324	1.138	1.164	87	1.528	1.199	1.274	119	1.256	1 169	1.074
57	1.314	0.990	1.328	88	1.305	1.191	1.096	120	1.709	1.476	1.157
58	1.208	1.073	1.126	89	1.290	1.027	1.256	121	1.619	1.302	1.244
59	1.412	1 274	1 109	90	1.421	1.267	1 121	122	1.647	1.549	1.063
60	1.772	1.437	1.233	91	1.312	1 166	1.126	123	1.456	1.250	1.164

Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min
124	1.874	1.738	1.078	155	1.726	1.416	1.219	186	1.629	1.433	1.136
125	1.728	1.420	1.217	156	1.530	1.246	1.228	187	1.433	1.256	1.141
126	1.676	1.431	1.171	157	1.594	1.504	1.060	188	1.496	1.223	1.223
127	1.455	1.137	1.279	158	1.631	1.367	1.193	189	1.371	1.201	1.141
128	1.624	1.527	1.063	159	1.382	1.266	1.092	190	1.466	1.276	1.148
129	1.484	1 193	1.244	160	1.697	1.554	1.092	191	1.597	1.494	1.069
130	1.616	1.430	1.130	161	2.068	1.590	1.301	192	1.683	1.512	1.113
131	1.531	1.296	1.182	162	1.794	1.455	1.233	193	1.524	1.390	1.097
132	1.537	1.182	1.301	163	1.639	1.580	1.038	194	1.550	1.325	1.169
133	1.480	1.301	1.138	164	1.672	1.529	1.094	195	1.466	1.269	1 155
134	1.543	1.269	1.216	165	1.431	1.329	1.077	196	1.412	1.264	1.118
135	1.638	1.491	1.099	166	2.086	1.651	1.264	197	1.586	1.535	1.033
136	1.482	1.261	1.175	167	1.994	1.653	1.206	198	1.439	1.403	1.025
137	1.511	1.289	1.172	168	1.688	1.564	1.079	190	1.663	1.369	1.215
138	1.452	1.229	1.181	169	1.595	1.247	1.279	191	1.620	1.498	1.081
139	1.625	1.615	1.006	170	1.789	1.675	1.068	192	1.563	1.432	1.091
140	2.019	1.807	1.117	171	1.521	1.258	1.209	193	1.247	1.095	1.138
141	1.414	1.016	1.393	172	1.261	1.160	1.087	194	1.589	1.486	1.069
142	1.763	1.457	1.210	173	1.453	1.445	1.006	195	1.622	1.452	1.117
143	1.483	1.288	1.151	174	1.402	1.108	1.266	196	1.824	1.739	1.049
144	1.533	1.317	1.164	175	1.626	1.456	1.116	197	1.449	1.251	1.158
145	1.378	1.133	1.216	176	1.530	1 154	1.326	198	1.521	1.339	1.136
146	1.961	1.584	1.238	177	1.395	1.292	1.080	199	1.886	1.702	1.108
147	1.606	1.411	1 139	178	1.506	1.259	1 196	200	1.798	1.550	1.160
148	1.796	1.642	1.093	179	1.411	1.324	1.066	201	1.866	1.278	1.460
149	1.482	1.277	1 160	180	1.308	1.184	1.105	202	1.876	1.642	1.143
150	1.488	1.295	1 149	181	1.367	1.067	1.281	203	1.504	1.298	1 159
151	1.300	1.233	1.054	182	1.618	1.292	1.253	204	1.624	1.217	1.334
152	1.896	1.511	1.255	183	1.375	1.270	1.083	205	1.610	1.338	1.203
153	1.378	1.176	1.172	184	1.409	1.280	1 101	206	1.552	1.254	1.237
154	1.507	1.308	1.152	185	1.182	1.103	1.072	207	1.689	1.461	1.156

Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min
208	1.611	1.521	1.059	240	1.516	1.425	1.064	271	1.453	1.340	1.084
209	1.389	1.342	1.035	241	1.189	0.895	1.328	272	1.317	1.005	1.311
210	1.406	1.309	1.074	242	1.512	1.373	1 101	273	1.404	1.007	1.393
211	1.701	1.236	1.376	243	1 147	0.779	1.473	274	1.800	1.569	1.147
212	1.654	1.538	1.075	244	1.474	1.314	1.122	275	1.650	1.441	1 145
213	1.718	1.418	1.211	245	1.247	1.076	1.159	276	1.502	1.184	1.269
214	1.844	1.689	1.091	246	1.369	1.229	1.114	277	1.633	1.429	1.142
215	1.788	1.475	1.212	247	1.482	1.448	1.024	278	1.645	1.612	1.020
216	1.578	1.276	1.236	248	1.367	1.028	1.330	279	1.535	1.167	1.315
217	1.681	1.276	1.317	249	1.314	1.113	1.181	280	1.604	1.516	1.058
218	1.605	1.431	1.121	250	1.538	1.208	1.273	281	1.479	1.383	1.070
219	1.680	1.479	1.136	251	1.474	1.291	1.141	282	1.347	1.249	1.078
220	1.419	1.353	1.049	252	1.499	1.367	1.096	283	1.468	1.450	1.013
221	1.460	1.305	1.118	253	1.568	1.352	1.160				
223	1.393	1.274	1.093	254	1.330	1.211	1.098				
224	1.373	1.274	1.077	255	1.610	1.371	1 174				
225	1.434	1.023	1.402	256	1.327	1.249	1.062				
226	1.708	1.435	1.191	257	1.366	1.228	1 112				
227	1.308	1.205	1.085	258	1.548	1.330	1 164				
228	1.252	1.221	1.025	259	1.331	1.120	1 188				
229	1.630	1.481	1.101	260	1.468	1.285	1.143				
230	1.397	1.101	1.268	261	1.437	1.233	1.165				
231	1.259	1.006	1.251	262	1.412	1.326	1.065				
232	1.420	1.296	1.096	263	1.451	1.222	1.188				
233	1.162	0.785	1.480	264	1.614	1.439	1.121				
234	1.288	1.239	1.039	265	1.421	1.385	1.026				
235	1.309	1.045	1.252	266	1.450	1.297	1.118				
236	1.360	1 116	1.219	267	1.346	1.071	1.257				
237	1.332	1.206	1.104	268	1.468	1.360	1.079				
238	1.325	1.226	1.080	269	1.217	1.021	1.191				
239	1.267	1.044	1.213	270	1.629	1.461	1.115				

Appendix 6. Raw data for 980625-4A cells grown at 25C in 120 g/L NaCl.
Measurements are in microns.

Cell Number	Major Axis	Minor Axis	Maj / Min	Average Major	Standard Dev Major	Average Minor	Standard Dev Minor	Avaerage Major:Minor	Standard Dev Major:Minor
1	1.416	1.277	1.109	1.650	0.194	1.477	0.155	1.117	0.060
2	1.820	1.667	1.092						
3	1.696	1.525	1.112						
4	1.705	1.542	1.106						
5	1.716	1.543	1.113						
6	1.638	1.464	1.119						
7	1.533	1.397	1.097						
8	1.751	1.544	1.134						
9	1.740	1.559	1.116						
10	1.686	1.584	1.064						
11	1.607	1.538	1.045						
12	1.575	1.547	1.018						
13	2.036	1.712	1.189						
14	1.911	1.465	1.305						
15	1.768	1.629	1.085						
16	1.937	1.597	1.213						
17	1.525	1.374	1.110						
18	1.549	1.444	1.072						
19	1.234	1.189	1.038						
20	1.658	1.447	1.146						
21	1.565	1.479	1.058						
22	1.523	1.402	1.087						
23	1.899	1.736	1.094						
24	1.528	1.390	1.100						
25	1.831	1.593	1.150						
26	1.601	1.382	1.159						
27	1.707	1.370	1.246						
28	1.644	1.477	1.113						
29	1.677	1.488	1.127						
30	1.065	0.935	1.139						
31	1.601	1.487	1.077						

Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min
32	1.620	1.512	1.072	65	1.600	1.430	1.118	98	1.467	1.320	1.112
33	1.913	1.593	1.201	66	1.803	1.710	1.054	99	1.555	1.314	1.183
34	1.638	1.190	1.377	67	1.739	1.259	1.381	100	1.309	1.268	1.033
35	1.587	1.442	1.101	68	1.577	1.406	1 122	101	1.491	1.477	1.010
36	1 154	0.981	1.176	69	2.242	1.540	1.456	102	1.271	1.163	1.093
37	1.368	0.991	1.381	70	1.854	1.610	1.151	103	1.381	1.240	1.113
38	1.656	1.424	1.163	71	1.661	1.608	1.033	104	1.723	1.457	1.182
39	1.589	1.096	1.450	72	1.721	1.586	1.085	105	1.593	1.573	1.013
40	1.339	1.170	1.145	73	2.083	1.632	1.277	106	1.554	1.479	1.051
41	1.316	1.036	1.270	74	1.809	1.479	1.223	107	1.742	1.615	1.079
42	1.473	1.201	1.226	75	2.088	1.524	1.370	108	1.726	1.426	1.210
43	1.760	1.616	1.089	76	1.905	1.776	1.073	109	1.665	1.512	1.102
44	1.598	1.242	1.287	77	1.575	1.420	1.109	110	1.530	1.188	1.287
45	1.385	1 198	1.156	78	1.779	1.560	1.140	111	1.162	0.981	1.184
46	1.369	1.226	1.117	79	1.158	1.043	1.111	112	1.685	1.470	1.146
47	1.691	1.535	1.102	80	1.327	1.047	1.268	113	1.765	1.518	1.163
48	1.586	1.341	1.183	81	1.361	1.094	1.243	114	1.527	1.466	1.042
49	1.563	1.492	1.048	82	1.643	1.548	1.061	115	1.835	1.725	1.064
50	1.333	1.263	1.055	83	1.370	1.121	1.222	116	1.600	1.367	1.171
51	1.224	1.145	1.069	84	1.681	1.467	1 147	117	1.618	1.528	1.058
52	1.626	1.248	1.303	85	1.906	1.727	1 104	118	1.680	1.493	1.125
53	1.495	1.337	1 118	86	1.336	1 125	1 188	119	1.493	1.475	1.012
54	1.762	1.513	1.165	87	1.439	1.072	1.343	120	1.658	1.428	1.161
55	1.870	1.788	1.046	88	1.548	1.454	1.065	121	1.437	1.282	1.121
56	1.640	1.493	1.098	89	1.221	0.999	1.222	122	1.679	1.422	1 180
57	1.578	1.501	1.051	90	1.793	1.539	1.165	123	1.499	1.218	1.230
58	1.718	1.433	1.199	91	1.814	1.660	1.093	124	1.508	1.437	1.049
59	1.726	1.602	1.077	92	1.374	1.283	1.071	125	1.434	1.264	1 134
60	1.719	1.440	1.194	93	1.600	1.513	1.057	126	1.816	1.660	1.094
61	1.856	1.444	1.285	94	1.641	1.245	1.318	127	1.705	1.428	1 194
62	1.447	1.280	1.130	95	1.410	1.305	1.081	128	1.712	1.535	1.115
63	1.512	1.401	1.080	96	1.539	1.332	1.155	129	1.726	1.491	1.157
64	1.291	1.145	1.127	97	1.355	1.143	1.186	130	1.401	1.131	1.239

Cell Number	Major Axis	Minor Axis	Maj / Min
131	2.022	1.717	1.178
132	1.429	1.336	1.070
134	1.495	1.346	1.111
135	1.779	1.560	1.141
136	1.976	1.774	1 114
137	1.547	1.536	1.007
138	2.190	1.729	1.266
139	1.575	1.318	1.195
140	1.785	1.595	1.119
141	1.741	1.646	1.058
142	1.479	1.236	1.196
143	1.666	1.501	1.110
144	1.520	1.450	1.049
145	1.709	1.385	1.234
146	1.067	0.989	1.079
147	1.293	1.109	1 166
148	1.405	1.169	1.202
149	1.520	1.293	1.175
150	1.484	1.180	1.258
151	1.440	1.224	1.176
152	1.527	1.458	1.048
153	1.690	1.618	1.045
154	1.351	1.117	1.209
155	1.261	1.156	1.091
156	1.270	1.177	1.079
157	1.574	1.379	1.141
158	1.197	0.987	1.212
159	1.598	1.313	1.217
160	1.991	1.764	1.129
161	1.376	1.310	1.051
162	1.673	1.548	1.081
163	1.312	1 102	1.191
164	1.170	1 110	1.054
165	1.348	1.162	1.160
166	1.274	1.031	1.235
167	1.156	1.091	1.059
168	1.262	0.986	1.280
169	1.488	1.365	1.091
170	1.309	1.277	1.025
171	1.237	1.186	1 043
172	1.612	1.375	1.172
173	1.292	1.180	1.095
174	1.616	1.395	1.158
175	1.492	1.304	1.144
176	1.163	1.103	1.054
177	1.609	1.409	1.141
178	1.466	1 145	1.280
179	1.817	1.667	1 090
180	1.413	1.133	1.247
181	1.201	0.986	1.218
182	1.520	1.366	1 113
183	1.783	1.496	1 192
184	1.456	0.977	1.491
184	1.283	1 115	1.151
185	1.490	1.309	1.138
186	1.343	1 170	1.148
187	1.009	0.917	1.100
188	1.050	0.864	1.215
189	1.665	1.491	1.117
190	1.747	1.346	1.298
191	1.500	1.304	1.151
192	1.652	1.487	1.111
193	1.721	1.470	1.170
194	1.490	1.353	1.101
195	1.653	1.523	1.085
196	1.485	1.423	1.043
197	1.543	1.191	1.295
198	1.651	1.572	1.051
199	1.376	1.291	1.066
200	1.915	1.713	1 118
201	2.211	1.523	1.452
202	2.343	1.750	1.339

Apendix 7 Raw data for 980625-4A cells grown at 40C in 0 g/L NaCl. Measurements are in microns. Measurements are in microns.

Cell Number	Major Axis	Minor Axis	Maj / Min	Average Major	Standard Dev Major	Average Minor	Standard Dev Minor	Average Major:Minor	Standard Dev Major:Minor
1	1.739	1.597	1.089	1.615	0.409	1.389	0.382	1.177	0.121
2	1.935	1.610	1.202						
3	1.158	0.917	1.263						
4	2.472	2.069	1.195						
5	1.186	0.979	1.211						
6	1.581	1.558	1.015						
7	1.388	1.356	1.023						
8	1.790	1.724	1.038						
9	1.920	1.517	1.266						
10	1.594	1.440	1.107						
11	1.669	1.453	1.149						
12	2.154	1.517	1.420						
13	1.271	0.960	1.324						
14	1.040	0.951	1.093						
15	1.214	1.011	1.201						
16	1.599	1.489	1.074						
17	1.104	1.054	1.047						
18	1.801	1.514	1.189						
19	2.417	2.249	1.074						
20	1.856	1.593	1.166						
21	1.754	1.314	1.335						
22	1.564	1.323	1.182						
23	1.444	1.319	1.095						
24	1.749	1.680	1.041						
25	2.185	1.723	1.268						
26	1.636	1.372	1.192						
27	1.324	1.134	1.168						
28	0.863	0.611	1.411						
29	1.543	1.070	1.441						
32	0.994	0.837	1.186						
31	2.131	2.114	1.008						

Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min
32	2.278	1.557	1.463	65	2.502	2.228	1.123
33	2.193	1.921	1.141	66	1.399	1.324	1.057
34	1.989	1.823	1.091	67	1.725	1.638	1.053
35	1.799	1.547	1.163	68	1.714	1.529	1.121
36	1.624	1.480	1.097	69	2.065	1.673	1.234
37	1.831	1.743	1.050	70	1.801	1.526	1.180
38	1.081	1.001	1.080	71	1.451	1.304	1.113
39	2.240	2.003	1.118	72	1.581	1.180	1.340
40	1.565	1.068	1.466	73	2.148	1.518	1.415
41	1.596	1.325	1.205	74	1.304	1.147	1.137
42	1.473	1.366	1.079	75	1.098	0.928	1 183
43	1.074	0.812	1.322	76	1.833	1.528	1.200
44	2.033	1.403	1.449	77	0.913	0.761	1.200
45	1.712	1.420	1.205	78	1.870	1.579	1 184
46	1.901	1.523	1.248	79	1.039	0.899	1 156
47	1.813	1.375	1.319	80	1.499	1.092	1.372
48	1.040	0.806	1.290	81	1.934	1.501	1.288
49	1.781	1.192	1.494	82	1.060	0.939	1.129
50	1.197	1.022	1.171	83	1.806	1.614	1.119
51	1.346	1.130	1.191	84	1.542	1.403	1.099
52	1.048	0.711	1.474	85	1.686	1.434	1.175
53	1 118	0.811	1.378	86	1.752	1.579	1.109
54	1 137	1.078	1.055	87	1.467	1.418	1.034
55	1.658	1.359	1.220	88	1.095	0.965	1.135
56	0.709	0.591	1.200	89	2.215	1.593	1.391
57	1.727	1.451	1.190	90	1.956	1.486	1.316
58	1.440	1.423	1.013	91	1.218	1.151	1.058
59	1.591	1.151	1.382	92	2.078	1.800	1.155
60	1.583	1.491	1.062	93	1.893	1.742	1.087
61	1.742	1.310	1.330	94	2.097	2.079	1.009
62	1.922	1.350	1.423	95	1.664	1.472	1.130
63	1.885	1.582	1.192	96	1.686	1.364	1.236
64	1.300	1.106	1.176	97	1.534	1.272	1.206

Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min
98	1.253	1.018	1.231	131	1.697	1.467	1.157
99	1.153	0.957	1.205	132	1.154	1.034	1.116
100	1.847	1.623	1.138	133	1.444	1.124	1.284
101	1.074	1.030	1.042	134	1.369	1.107	1.238
102	1.409	1.023	1.378	135	2.512	1.899	1.323
103	1.571	1.287	1.221	136	1.195	1.069	1.117
104	1.501	1.488	1.009	137	1.042	1.032	1.010
105	1.060	0.888	1.194	138	1.388	1.312	1.058
106	1.669	1.278	1.306	139	1.190	1.031	1.154
107	1.696	1.355	1.251	140	1.853	1.379	1.344
108	1.168	1.084	1.077	141	1.751	1.584	1.106
109	1.979	1.858	1.065	142	1.287	1.090	1.181
110	1.782	1.565	1.139	143	1.166	0.996	1.170
111	1.005	0.826	1.217	144	1.513	1.374	1.101
112	1.133	0.953	1.189	145	1.532	1.324	1.157
113	1.910	1.687	1.132	146	1.489	1.087	1.370
114	2.002	1.405	1.425	147	1.177	0.877	1.342
115	1.176	1.036	1.136	148	1.195	1.088	1.099
116	1.740	1.452	1.199	149	0.961	0.887	1.084
117	1.392	1.001	1.391	150	2.270	1.702	1.333
118	1.107	0.912	1.213				
119	1.302	1.086	1.199				
120	1.356	1.249	1.085				
121	1.234	0.957	1.289				
122	1.752	1.656	1.058				
123	1.400	1.263	1.108				
124	2.375	2.172	1.094				
125	1.428	1.276	1.119				
126	1.298	1.189	1.092				
127	2.002	1.696	1.180				
128	1.639	1.405	1.167				
129	2.157	1.900	1.136				
130	1.726	1.425	1.211				

Appendix 8. Raw data for 980625-4A cells grown at 40C in 40 g/l NaCl.
Measurements are in microns.

Cell Number	Major Axis	Minor Axis	Maj / Min	Average Major	Standard Dev Major	Average Minor	Standard Dev Minor	Average Major:Minor	Standard Dev Major:Minor
1	1.524	1.117	1.364	1.486	0.213	1.263	0.163	1.181	0.118
2	1.189	1.146	1.038						
3	1.235	0.835	1.479						
4	1.532	1.317	1.163						
5	1.208	1.091	1.108						
6	1.249	1.052	1.187						
7	1.467	1.413	1.038						
8	1.415	1.291	1.096						
9	1.487	1.386	1.073						
10	1.726	1.411	1.223						
11	1.251	1.067	1.173						
12	1.599	1.441	1.110						
13	1.684	1.334	1.262						
14	1.364	1.130	1.208						
15	1.269	1.246	1.018						
16	1.712	1.431	1.197						
17	1.319	1.117	1.181						
18	1.469	1.130	1.300						
19	1.340	1.254	1.069						
20	1.606	1.270	1.265						
21	1.992	1.658	1.201						
22	1.380	1.231	1.121						
23	1.617	1.193	1.355						
24	1.538	1.347	1.141						
25	1.869	1.498	1.247						
26	1.269	1.205	1.053						
27	1.880	1.302	1.444						
28	1.316	1.168	1.126						
29	1.538	1.430	1.075						
30	1.680	1.387	1.211						
31	1.339	1.249	1.071						

Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min
32	1.254	1.013	1.238	65	1.623	1.458	1.114	98	1.121	0.998	1.124
33	1.279	0.873	1.466	66	1.912	1.523	1.255	99	1.606	1.453	1.105
34	0.800	0.722	1.108	67	1.607	1.313	1.225	100	1.740	1.346	1.293
35	1.359	1.237	1.099	68	1.742	1.572	1.109	101	1.612	1.444	1.116
36	1.561	1.201	1.299	69	2.042	1.596	1.280	102	1.397	1.244	1.123
37	1.120	0.914	1.225	70	2.167	1.816	1 193	103	1.509	1.238	1.219
38	1.128	0.753	1.499	71	1.690	1.455	1 162	104	1.799	1.395	1.290
39	1.391	1.283	1.085	72	1.653	1.584	1.044	105	1.814	1.603	1 131
40	0.832	0.813	1.023	73	1.667	1.456	1 145	106	1.353	1.134	1 193
41	1 148	1.007	1.140	74	1.509	1.323	1.141	107	1.257	1.186	1.060
42	1.269	1.080	1.176	75	1.508	1.441	1.047	108	1.370	1.107	1.238
43	1.393	1.292	1.078	76	1.917	1.680	1.141	109	1.397	1.141	1.225
44	1.612	1.193	1.351	77	1.758	1.572	1.118	110	1.340	1.153	1.162
45	1.645	1.493	1.102	78	1.516	1.362	1.113	111	1.063	0.779	1.365
46	1.584	1.126	1.407	79	1.495	1.348	1.109	112	1.706	1.408	1.211
47	1.566	1.412	1.109	80	1.395	1.265	1.103	113	1.766	1.434	1.232
48	1.525	1.312	1.163	81	1.877	1.349	1.391	114	1.219	0.910	1.340
49	1.508	1.304	1 156	82	1.848	1.820	1.015	115	1.071	0.964	1.112
50	1.916	1.292	1.484	83	1.898	1.444	1.314	116	1.257	1.013	1.241
51	1.562	1.439	1.086	84	2.087	1.723	1.212	117	1.546	1.368	1.130
52	2.077	1.541	1.348	85	1.372	1.198	1 145	118	1.053	1.012	1.040
53	1.433	1.356	1.057	86	1.801	1.227	1.468	119	1.647	1.382	1.192
54	1.352	1.307	1.034	87	1.830	1.736	1.054	120	1.406	1.227	1.146
55	1.471	1.351	1.089	88	1.453	1.266	1 147	121	1.278	1.012	1.263
56	1.675	1.364	1.229	89	1.563	1.341	1.166	122	1.502	1.272	1.181
57	1.596	1.447	1.103	90	1.844	1.390	1.326	123	1.507	1.343	1.122
58	1.445	1.205	1.200	91	1.593	1.520	1.048	124	1.424	1.366	1.042
59	1.877	1.679	1.118	92	1.734	1.274	1.361	125	1.302	1.192	1.092
60	1.707	1.518	1.124	93	1.872	1.684	1 112	126	1.268	1.068	1.187
61	1.506	1.302	1.156	94	1.177	1 117	1.053	127	1.805	1.521	1.187
62	1.507	1.316	1 145	95	1.539	1.360	1.132	128	1.391	1.086	1.281
63	1.556	1.537	1.013	96	1.312	1 159	1.132	129	1.406	1.404	1.001
64	1.614	1.324	1.220	97	1.734	1.460	1.188	130	1.278	1.217	1.050

Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min
131	1.213	1.083	1.120	164	1.070	1.034	1.034	197	1.092	0.860	1.269
132	1.340	1.043	1.285	165	1.399	1.319	1.061	198	1.234	0.932	1.324
133	1.470	1.365	1.077	166	1.006	0.861	1.169	199	1.220	0.883	1.382
134	1.798	1.495	1.203	167	1.439	1.238	1.162	200	1.588	1.341	1.184
135	1.451	1.356	1.070	168	1.355	1.175	1.153	201	1.104	0.758	1.455
136	1.324	1.162	1.140	169	1.401	1.066	1.314	202	1.652	1.298	1.273
137	1.438	1.328	1.083	170	1.293	1.065	1.213	203	1.604	1.158	1.385
138	1.325	1.124	1.179	171	1.425	1.320	1.079	204	1.733	1.262	1.373
139	1.513	1.407	1.075	172	1.834	1.332	1.376	205	1.602	1.415	1.132
140	1.594	1.493	1.067	173	1.414	1.239	1.141	206	1.555	1.170	1.328
141	1.519	1.375	1.105	174	1.745	1.536	1.136	207	1.703	1.457	1.169
142	1.408	1.227	1.147	175	1.898	1.429	1.328	208	1.592	1.435	1.110
143	1.529	1.391	1.099	176	1.381	1.255	1.100	209	1.754	1.606	1.092
144	1.783	1.604	1.112	177	1.106	0.938	1.179	210	1.433	1.356	1.057
145	1.394	1.301	1.072	178	1.151	1.053	1.093	211	1.078	0.848	1.271
146	1.345	1.129	1.192	179	1.477	1.252	1.179	212	1.535	1.523	1.008
147	1.433	1.238	1.158	180	1.489	1.203	1.238	213	1.325	1.263	1.049
148	1.070	0.972	1.100	181	1.465	1.404	1.043	214	1.394	1.040	1.340
149	1.353	1.107	1.222	182	1.712	1.499	1.142	215	1.635	1.191	1.373
150	1.384	1.077	1.285	183	1.768	1.454	1.216	216	1.447	1.153	1.255
151	1.535	1.418	1.083	184	1.363	1.217	1.120	217	1.573	1.390	1.132
152	1.322	1.200	1.102	185	1.897	1.560	1.216	218	1.813	1.275	1.422
153	1.611	1.458	1.105	186	1.509	1.291	1.170	219	1.513	1.303	1.161
154	1.306	1.191	1.096	187	1.706	1.536	1.111	220	1.370	1.210	1.132
155	1.703	1.521	1.119	188	1.957	1.707	1.146	221	1.661	1.433	1.159
156	1.541	1.397	1.103	189	1.529	1.214	1.259	222	1.561	1.437	1.087
157	1.626	1.408	1.155	190	1.265	1.056	1.198	223	1.459	1.263	1.155
158	1.542	1.258	1.226	191	1.579	1.499	1.053	224	1.564	1.205	1.298
159	1.562	1.234	1.266	192	1.465	1.221	1.200	225	1.383	1.180	1.172
160	2.207	1.825	1.209	193	1.110	1.047	1.059	226	1.607	1.508	1.065
161	1.783	1.654	1.078	194	1.560	1.433	1.089	227	1.356	1.257	1.078
162	1.455	1.346	1.081	195	1.239	1.092	1.135	228	1.728	1.447	1.194
163	1.547	1.156	1.338	196	1.329	1.169	1.138	229	1.880	1.589	1.183

Cell Number	Major Axis	Minor Axis	Maj / Min
230	1.530	1.270	1.204
231	1.376	1.192	1.155
232	1.314	1.148	1.144
233	1.490	1.428	1.044
234	1.695	1.667	1.017
235	1.609	1.391	1.156
236	1.648	1.577	1.045
237	1.767	1.695	1.042
238	1.817	1.649	1.102
239	1.271	1.198	1.061
240	1.785	1.631	1.095
241	1.515	1.399	1.083
242	2.172	1.844	1.178
243	1.678	1.523	1.102
244	1.691	1.633	1.036

Appendix 9. Raw data for 980625-4A cells grown at 40C in 80 g/L NaCl.
Measurements are in microns

Cell Number	Major Axis	Minor Axis	Maj / Min	Average Major	Standard Dev Major	Average Minor	Standard Dev Minor	Average Major:Minor	Standard Dev Major:Minor
1	1.245	1.005	1.239	1.400	0.219	1.173	0.208	1.201	0.105
2	0.891	0.783	1.139						
3	1.099	0.859	1.279						
4	1.450	0.975	1.487						
5	1.300	0.946	1.374						
6	1.466	1.151	1.274						
7	1.589	1.364	1.165						
8	1.480	1.460	1.014						
9	1.737	1.399	1.242						
10	1.383	1.072	1.290						
11	1.530	1.259	1.216						
12	1.422	1.241	1.146						
13	1.660	1.523	1.090						
14	1.326	1.166	1.138						
15	1.273	1.228	1.037						
16	1.451	1.188	1.222						
17	1.496	1.233	1.213						
18	1.258	1.007	1.249						
19	1.319	1.077	1.224						
20	1.626	1.392	1.168						
21	1.541	1.193	1.292						
22	1.437	1.328	1.082						
23	1.600	1.393	1.148						
24	1.755	1.419	1.237						
25	1.718	1.534	1.120						
26	1.135	1.078	1.053						
27	1.543	1.260	1.224						
28	1.218	1.108	1.100						
29	1.004	0.811	1.238						
30	1.036	0.904	1.146						
31	1.400	1.007	1.390						

Cell Number	Major Axis	Minor Axis	aj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min
32	1.408	1.228	1.147	65	1.229	1.049	1.172	98	1.420	1.266	1.122
33	1.159	1.017	1.140	66	1.137	1.037	1.096	99	1.708	1.386	1.232
34	1.449	1.381	1.049	67	1.596	1.281	1.246	100	0.903	0.863	1.046
35	1.355	1.242	1.091	68	1.684	1.439	1.171	101	1.117	1.048	1.066
36	1.721	1.476	1.166	69	1.466	1.421	1.032	102	1.341	1.097	1.223
37	1.458	1.254	1 163	70	1.732	1.263	1.371	103	1.330	1.171	1.136
38	1.098	0.958	1.146	71	1.796	1.358	1.322	104	1.701	1.549	1.098
39	1.258	1.107	1.137	72	1.100	0.987	1.114	105	1.482	1.291	1.148
40	1.451	1.281	1.132	73	1.347	1.141	1 180	106	1.647	1.563	1.054
41	1.479	1.253	1 180	74	1.380	1.335	1.033	107	1.599	1.221	1.310
42	1.389	1.333	1.042	75	1.599	1.382	1.157	108	1.470	1.159	1.268
43	1.322	1.169	1.130	76	1.394	1.240	1.125	109	1.583	1.383	1.145
44	1.678	1.402	1.197	77	1.244	1.099	1.132	110	1.779	1.577	1.128
45	1.529	1.290	1.185	78	1.412	0.945	1.495	111	1.151	1.076	1.070
46	1.440	1.275	1.129	79	1.393	1.166	1.195	112	1.417	1.253	1 131
47	1.458	1.259	1.158	80	1.339	1.229	1.089	113	1.327	1.270	1.045
48	1.612	1.263	1.276	81	1.065	0.813	1.311	114	0.954	0.859	1.110
49	1.632	1.425	1 146	82	1.449	1.386	1.045	115	1.547	1.393	1.111
50	1.106	0.806	1.371	83	1.323	0.952	1.389	116	0.983	0.793	1.240
51	1.075	0.835	1.287	84	1.442	1.249	1 155	117	1.307	1.080	1.210
52	1.715	1.314	1.305	85	1.488	1.259	1.182	118	1.233	1 100	1.121
53	1.275	1.146	1.113	86	1.870	1.435	1.303	119	1.334	1.085	1.230
54	0.846	0.839	1.008	87	1.546	1.401	1.104	120	1 130	1.024	1.104
55	1.346	1.327	1.015	88	1.594	1.585	1.006	121	1.475	1.321	1.117
56	1.393	1.272	1.094	89	1.435	1.379	1.041	122	1.355	1.262	1.074
57	1.590	1.459	1.090	90	1.562	1.410	1.108	123	1.536	1.290	1.191
58	1.402	1.160	1.209	91	1.657	1.517	1.092	124	1.435	1.245	1.153
59	1.125	0.934	1.204	92	1.534	1.435	1.068	125	1.529	1.335	1.145
60	1.300	1.096	1.186	93	1.540	1.281	1.202	126	1 093	1.030	1.061
61	1.288	1.199	1.074	94	1.452	1.311	1.107	127	1.201	1.132	1.061
62	1.571	1.220	1.288	95	1.296	1.111	1.166	128	1.479	1.366	1.083
63	1.256	1.168	1.075	96	1.420	1.158	1.226	129	1.548	1.176	1.317
64	1.730	1.356	1.276	97	1.430	1.171	1.221	130	1.477	1.289	1.146

Cell Number	Major Axis	Minor Axis	aj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min
131	1.635	1.350	1.211	164	1.719	1.413	1.216	197	1.574	1.316	1.196
132	1.569	1.413	1.110	165	1.581	1.141	1.386	198	1.712	1.239	1.381
133	1.330	1.167	1.139	166	1.501	1.106	1.357	199	1.585	1.209	1.311
134	1.445	1.211	1.193	167	1.480	1.222	1.212	200	1.745	1.474	1.184
135	1.290	1.150	1.122	168	1.443	1.261	1.144	201	2.170	1.823	1.190
136	1.414	1.232	1.148	169	1.607	1.198	1.341	202	1.860	1.353	1.375
137	1.573	1.296	1.214	170	0.819	0.810	1.011				
138	1.365	1.324	1.030	171	1 131	0.967	1.170				
139	1.546	1.197	1.291	172	1.526	1.216	1.255				
140	1.545	1.389	1.112	173	1.209	1.000	1.209				
141	1.089	1.024	1.064	174	1.749	1.320	1.326				
142	1.379	1.000	1.380	175	1.697	1.450	1 171				
143	1.413	1.249	1.131	176	1.592	1.486	1.071				
144	1.236	1.185	1.043	177	1.331	1.184	1.124				
145	1.314	1.234	1.065	178	1.448	1.232	1.175				
146	1.526	1.176	1.298	179	1.324	1.243	1.065				
147	1.428	1.160	1.232	180	1.432	1.239	1.156				
148	1.551	1.515	1.024	181	1.543	1.412	1.093				
149	1.629	1.507	1.080	182	1.870	1.541	1.213				
150	1.572	1.203	1.306	183	1.634	1.397	1 170				
151	1.157	1.018	1.136	184	1.238	1.072	1.155				
152	1.283	1.120	1.145	185	1.523	1.252	1.217				
153	1.435	1.291	1.111	186	1.302	1.270	1.025				
154	1.117	0.781	1.429	187	1.552	1.173	1.323				
155	1.513	1.419	1.066	188	1.575	1.460	1.079				
156	1.594	1.353	1.178	189	1.415	1 165	1.215				
157	1.367	1.345	1.016	190	1.636	1.602	1.021				
158	1.556	1.322	1 177	191	1.374	1.203	1.142				
159	1.461	1.413	1.034	192	1.597	1.303	1.225				
160	1.431	1.324	1.080	193	1.531	1.420	1.079				
161	1.665	1.388	1.200	194	1.896	1.566	1.211				
162	1.568	1.492	1.051	195	1.326	1.143	1.160				
163	1.602	1.103	1.453	196	1.489	1.230	1.211				

Appendix 10. Raw data for 980625-4A cells grown at 40C in 120 g/L NaCl.
Measurements are in microns.

Cell Number	Major Axis	Minor Axis	Maj / Min	Average Major	Standard Dev Major	Average Minor	Standard Dev Minor	Average Major:Minor	Standard Dev Major:Minor
1	1.216	0.825	1.474	1.128	0.222	0.974	0.244	1.177	0.123
2	1.152	0.964	1.195						
3	1.739	1.657	1.050						
4	1.270	1.125	1.129						
5	1.099	0.988	1.112						
6	1.080	0.815	1.324						
7	0.807	0.612	1.318						
8	1.247	1.196	1.042						
9	0.981	0.731	1.342						
10	1.303	1.146	1.137						
11	1.302	1.210	1.076						
12	0.882	0.849	1.038						
13	1.342	1.134	1.184						
14	1.045	0.939	1.112						
15	1.259	1.205	1.044						
16	1.219	0.902	1.351						
17	1.082	0.930	1.164						
18	1.364	1.283	1.063						
19	1.246	1.107	1.125						
20	1.032	0.846	1.220						
21	1.082	0.969	1.116						
22	0.712	0.637	1.118						
23	0.975	0.699	1.396						
24	1.024	0.880	1.164						
25	0.931	0.821	1.134						
26	0.993	0.832	1.194						
27	1.004	0.682	1.472						
28	1.579	1.460	1.081						
29	1.454	1.310	1.110						
30	0.857	0.716	1.196						
31	1.119	1.050	1.065						
32	0.924	0.858	1.077						
33	0.909	0.753	1.208						

Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min
34	1.432	1.176	1.218	67	1.400	1.222	1 145	100	1.222	0.836	1.462
35	1.035	0.863	1.199	68	1.003	0.860	1.166	101	1.257	1.205	1.043
36	0.951	0.879	1.082	69	1.111	0.961	1.156	102	0.930	0.772	1.204
37	1.137	1.021	1.114	70	1.429	1.319	1.084	103	0.888	0.650	1.367
38	0.771	0.695	1 110	71	0.879	0.697	1.261	104	1.183	0.922	1.283
39	1.002	0.946	1.059	72	1.533	1.375	1.115	105	1.002	0.853	1.176
40	1.090	0.856	1.273	73	1.468	1.241	1.183	106	1.152	0.929	1.240
41	1.260	1.201	1.049	74	1.402	1.243	1.127	107	1.608	1.168	1.377
42	1.310	1.211	1.082	75	1.421	1.041	1.365	108	1.462	1.046	1.397
43	0.988	0.824	1 199	76	1.687	1.210	1.394	109	0.868	0.778	1.115
44	0.969	0.928	1.045	77	1.502	1.262	1.190	110	1.106	1.087	1.018
45	0.857	0.784	1.092	78	1.368	1.203	1.137	111	1.236	1.065	1.161
46	1.163	1.144	1.017	79	1.211	1.037	1.168	112	1.152	0.830	1.389
47	1.098	0.942	1 165	80	1.177	1.031	1.142	113	0.827	0.669	1.236
48	1.361	1.171	1 162	81	1.418	1.203	1.179	114	1.093	1.011	1.081
49	0.866	0.818	1.059	82	0.953	0.885	1.076	115	1.126	1.057	1.065
50	1.150	1.062	1.083	83	0.710	0.517	1.374	116	1.272	1.109	1.146
51	0.939	0.904	1.039	84	1.110	0.789	1.407	117	1.120	0.862	1.299
52	1.198	1.095	1.094	85	1.193	0.924	1.291	118	0.853	0.698	1.222
53	1.774	1.653	1.074	86	0.791	0.754	1.048	119	1.411	1.003	1.407
54	1.246	1 175	1.061	87	1.217	1.087	1.119	120	1.268	1.025	1.237
55	1.301	0.877	1.483	88	1.036	0.774	1.339	121	1.284	1.234	1.041
56	1.440	1.328	1.084	89	1.250	1.112	1.123	122	1.147	1.061	1.082
57	1.582	1.489	1.062	90	0.875	0.851	1.028	123	0.940	0.667	1.410
58	1.127	0.934	1.207	91	1.051	1.036	1.015	124	1.124	0.833	1.350
59	1.352	1.091	1.240	92	1.205	1.067	1.130	125	1.310	1.156	1.133
60	1.384	1 187	1.166	93	1.206	1.091	1.106	126	1.547	1.226	1.262
61	1.258	1 161	1.083	94	1.483	1.044	1.420	127	1.240	1 135	1.092
62	1 190	0.944	1.261	95	1.453	1.428	1.017	128	1.082	0.806	1.343
63	1.512	1.327	1.140	96	1.223	1.140	1.073	129	1.101	0.940	1.172
64	1.245	0.992	1.255	97	1.031	0.875	1.178	130	1.083	1.046	1.036
65	1 114	0.993	1 121	98	1.030	0.899	1 146	131	1.165	0.801	1.455
66	1 179	1.111	1.061	99	1.338	1.170	1.143	132	0.966	0.647	1.492

Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	aj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min
133	0.996	0.977	1.020	166	1.073	0.940	1.141	199	1.570	1.406	1.116
134	1.444	1.369	1.054	167	0.981	0.888	1.104	200	1.169	1.058	1.105
135	0.755	0.639	1 182	168	0.721	0.693	1.040	201	1.307	1.153	1.134
136	0.809	0.604	1 339	169	1.312	1.104	1.188	202	1.211	1.092	1.109
137	0.890	0.634	1.402	170	1.029	0.996	1 033	203	1.302	1.135	1.148
138	1.291	1.251	1.032	171	0.883	0.590	1.496	204	0.906	0.626	1.446
139	1.440	1 123	1.282	172	1.087	0.969	1.121	205	1.085	0.979	1.108
140	1.075	0.819	1.313	173	0.939	0.843	1.113	206	1.178	1.117	1.055
141	1.478	1.451	1.019	174	1.284	1.203	1.067	207	1.074	1.003	1.071
142	1.364	1.240	1.099	175	1.458	1.369	1.065	208	1.288	1.163	1.108
143	1.275	1.186	1.075	176	0.940	0.831	1.130	209	1.352	1.085	1.246
144	1.265	1 188	1.064	177	1.613	1.556	1.037	210	1.315	1.280	1.027
145	0.734	0.647	1.135	178	1.216	1.121	1.084	211	1.065	0.851	1.251
146	0.749	0.695	1.078	179	0.961	0.944	1.018	212	1.158	1.081	1.071
147	1.046	0.848	1.233	180	1.624	1.465	1.109	213	1.463	1.259	1.161
148	1.064	0.902	1 180	181	0.860	0.808	1.064	214	1.606	1.489	1.078
149	1.071	0.953	1 123	182	1.345	1.203	1.118	215	1.175	1 119	1.050
150	0.784	0.660	1 188	183	1.098	0.804	1.365	216	1.559	1.318	1.182
151	1.281	1.248	1.026	184	1.711	1.466	1 167	217	1.495	1.375	1.088
152	1.511	1.236	1.223	185	1.182	1.089	1.085	21	1.315	1.280	1.028
153	0.791	0.732	1.081	186	1.192	0.995	1.198	8	1.055	1.003	1.053
154	0.982	0.896	1.096	187	0.948	0.884	1.072	219	1.064	0.993	1.070
155	1.149	1.019	1.127	188	1.217	1.170	1.039	220	1.065	0.951	1.120
156	0.860	0.757	1.136	189	1.690	1.602	1.055	221	1.378	1.338	1.031
157	0.976	0.847	1.152	190	1.620	1.483	1.092	222	1.599	1.578	1.013
158	0.997	0.959	1.040	191	1.242	0.959	1.295	223	1 106	0.851	1.300
159	0.821	0.789	1.039	192	1.377	1.233	1.117	224	1.460	1.274	1.146
160	1.082	0.873	1.240	193	1.066	0.992	1.075	225	0.999	0.827	1 208
161	1.035	0.874	1.185	194	1.620	1.483	1.092	226	1.227	1.150	1.067
162	0.883	0.701	1.261	195	1.242	0.959	1.295	227	1.588	1.467	1.082
163	0.962	0.680	1.415	196	1.377	1.233	1.117	228	1.257	1.026	1.225
164	0.988	0.881	1.122	197	1.066	0.992	1.075	229	1.407	1.310	1.074
165	1.025	0.694	1.476	198	1.386	1.311	1.057	230	1.247	1.088	1.146

Cell Number	Major Axis	Minor Axis	Maj / Min	Cell Number	Major Axis	Minor Axis	Maj / Min
231	1.198	0.939	1.276	264	1.095	1.025	1.069
232	1.250	1.187	1.053	265	1.144	1.074	1.066
233	1.294	1.228	1.054	266	1.423	1.307	1.088
234	1.305	1.155	1.130	267	1.278	0.898	1.423
235	1.065	0.844	1.262	268	1.858	1.799	1.033
236	1.194	1.083	1.102	269	1.224	0.958	1.278
237	1.042	0.900	1.158	270	1.525	1.123	1.358
238	0.905	0.893	1.014	271	1.208	1.101	1.097
239	1.292	1.175	1.100	272	1.001	0.724	1.382
240	1.171	1.096	1.069	273	1.051	1.025	1.026
241	1.563	1.373	1.138	274	0.692	0.565	1.226
242	1.333	1.322	1.008	275	1.068	0.933	1.145
243	1.573	1.215	1.294	276	1.094	0.872	1.254
244	1.931	1.442	1.339	277	1.233	1.179	1.046
245	1.772	1.607	1.103	278	1.235	1.074	1.150
246	1.072	0.991	1.081	279	1.067	0.917	1.163
247	1.261	1.105	1.141	280	1.017	0.739	1.376
248	1.111	0.958	1.160	281	1.187	1.031	1.151
249	0.967	0.890	1.087	282	1.119	0.860	1.301
250	1.294	1.130	1.145	283	1.200	0.902	1.330
251	1.514	1.386	1.092	284	0.837	0.778	1.076
252	1.502	1.314	1.144				
253	1.283	1.255	1.023				
254	1.000	0.967	1.034				
255	1.137	0.884	1.287				
256	1.064	1.009	1.055				
257	1.421	1.197	1.187				
258	0.884	0.769	1.150				
259	1.244	1.181	1.053				
260	1.177	1.038	1.134				
261	0.926	0.904	1.025				
262	0.664	0.628	1.057				
263	1.083	0.820	1.321				

VITA

Janice Lynn Hironaka

Candidate for the Degree of Master of Science

Thesis: CHACTERIZATION OF A UNICELLULAR COCCOID GREEN ALGA COLLECTED FROM THE SALT PLAINS NATIONAL WILDLIFE REFUGE, OKLAHOMA

Major Field: Botany

Biographical:

Personal Data: Born in Sacramento, California, USA. August 6, 1953 to Elaine H. Hironaka and Jim M. Hironaka.

Education: Graduated from McClatchy Senior High School, Sacramento, California, 1971; Sacramento City College, Sacramento, California, 1986; California State University-Sacramento, Sacramento, California, 1995. Completed the requirements for the Master of Science degree with a major in Botany at Oklahoma State University, Stillwater, Oklahoma in December 2000.

Experience: Laboratory Teaching Assistant, California State University-Sacramento, Sacramento, California 1994 and 1995; Volunteer Tutor for the Athletic Department and the Students with Disabilities Program, California State University-Sacramento, Sacramento, California 1994-5; Volunteer Tutor for the Multicultural Development and Assessment Center, Oklahoma State University, Stillwater, Oklahoma 1996 to the present; Teaching Assistant for the National Air and Space Administration-American Indian Science and Technology Educational Consortium, Oklahoma State University, Stillwater, Oklahoma 1997; Lead Instructor, Biology Laboratory, for the National Air and Space Administration-American Indian Science and Technology Educational Consortium, Oklahoma State University, Stillwater, Oklahoma 1998; Teaching Assistant, Oklahoma State University, Stillwater, Oklahoma 1996-8; Teaching Assistant for the Oklahoma Principal's Science Scholars, Oklahoma State University, Stillwater, Oklahoma 1999.

Professional Memberships: National Association of Biology Teachers, Oklahoma Academy of Science, Phycological Society of America.

Honors and Awards: Gold Pin Award, Girl Scouts of the United States of America, Santa Rosa, California 1987; Outstanding Service Award, Girl Scouts of the United States of America, Santa Rosa, California 1988; Women Helping Women Award; Soroptimist International, Petaluma, California, 1988; President's Graduate Fellowship, Oklahoma State University, Stillwater 1997; Certificate of Achievement for Exceptional Leadership Excellence, Multicultural Development and Assessment Center, Oklahoma State University, Stillwater, Oklahoma 1997, 1998 and 1999; Outstanding Teaching Assistant, College of Arts and Science, Oklahoma State University, Stillwater, Oklahoma 1997; McAlester Scottish Rite Foundation Fellowship, Department of Botany, Oklahoma State University, Stillwater, Oklahoma 1998; McPherson Fund Fellowship, Department of Botany, Oklahoma State University, Stillwater, Oklahoma 1998-1999; Presidential Fellowships for Water, Energy, and the Environment, Environmental Institute, Oklahoma State University, Stillwater, Oklahoma 1999-2000 and 2000-2001 academic year.

Seminar Presentations:

Fun and Games with Rubber Tubing. 8th Annual Graduate Student Research Symposium, Oklahoma State University, Stillwater, Oklahoma 1997.

UV exposure of Several Marine Macroalgae. Department of Botany, Oklahoma State University, Stillwater, Oklahoma 1997.

Iron. Department of Botany, Oklahoma State University, Stillwater, Oklahoma 1997.

Description of a green microalga from the Great Salt Plains National Wildlife Refuge, Oklahoma. Oklahoma Academy of Science 88th Annual Technical Meeting, Oklahoma City, Oklahoma 1999.

Poster Presentations:

Extremophilic Algae and Cyanobacteria from the Great Salt Plains National Wildlife Refuge. XVI International Botanical Congress, St. Louis, Missouri, 1998.

Tolerance to Salinity and Temperature Stress in a Newly Discovered Green Alga from the Salt Plains National Wildlife Refuge. 3rd Annual Meeting, Environmental Institute, Oklahoma State University, Stillwater, Oklahoma 2000.