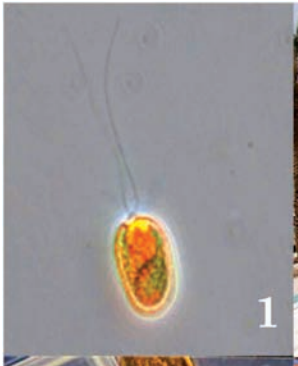
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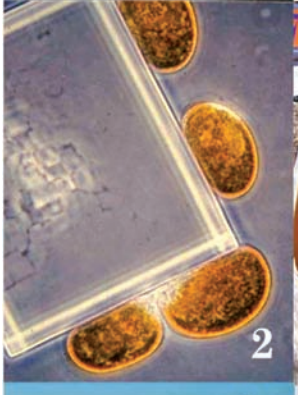
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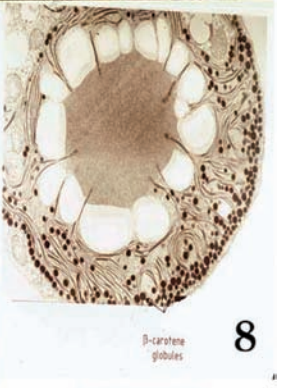
β-carotene
globules
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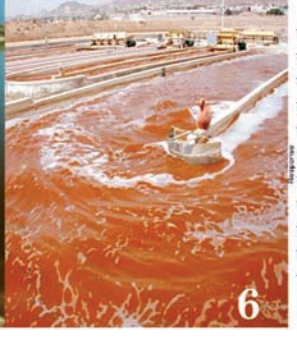
3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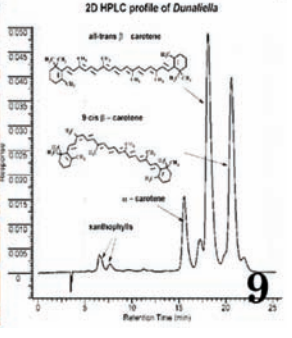
2D HPLC profile of Dunaliella
all-trans β - carotene
9-cis β – carotene
α – carotene
xanthophylls
Retention Time (min)
9

Front Cover shows: Cells of *Dunaliella bardawil* loaded with β catotene.

Frontipiece 1

Figure 1: Stressed cells of *Dunaliella bardawil*.

Figure 2: Cells of *Dunaliella bardawil* next to a salt crystal within a concentrated brine solution.

Figure 3: Photograph of a typical hypersaline habitat in which *Dunaliella* algae are found in the San Francisco Bay, USA.

Figure 4: Production facility of NBT with high-rate algae ponds in Eilat, Israel.

Figure 5: Small experimental raceway pond containing *Dunaliella bardawil.*

Figure 6: Close-up view of a large raceway pond with *Dunaliella bardawil* at NBT in Eilat, Israel.

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Figure 9: Shown is a two-dimensional HPLC profile obtained from orange cells of *Dunaliella bardawil*.

Photo credits: # 3 Dr. J. Polle; #1,2,4,5,6,7,8, and 9 Dr. Ami Ben-Amotz

Back Plate

Figure 1: A *Dunaliella salina* cell from a salt lake in Australia.

Figure 2: Stressed cells of *Dunaliella bardawil.*

Figure 3: Cells of *Dunaliella bardawil* next to a salt crystal within a concentrated brine solution.

Figure 4: Non-stressed green cells of *Dunaliella bardawil.*

Figure 5: Small experimental raceway pond containing *Dunaliella bardawil*.

Figure 6: 100 m^2 raceway pond with *Dunaliella bardawil* at NBT in Eilat, Israel.

Figure 7: Production facility of NBT with high-rate algae ponds in Eilat, Israel.

Figure 8: Photograph of a typical hyper-saline habitat in which *Dunaliella* thrive in the Great Salt Lake in Utah, USA.

Photo credits: #1 Dr. M. Dyall-Smith; #2,3,4,5,6, and 7 Dr. Ami Ben-Amotz; #8 Dr. J. Polle.

The Alga *Dunaliella*

Biodiversity, Physiology , Genomics and Biotechnology

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Published by Science Publishers, Enfield, NH, USA
An imprint of Edenbridge Ltd., British Channel Islands
Printed in India

ISBN 978-1-57808-545-3 (hardcover)

Library of Congress Cataloging-in-Publication Data

The alga Dunaliella : biodiversity, physiology, genomics and biotechnology/
editors, Ami Ben-Amotz, Jürgen E.W. Polle, D.V. Subba Rao. — 1st ed.
p. cm.
Includes bibliographical references and index.
ISBN 978-1-57808-545-3 (hardcover)
1. Dunaliella. I. Ben-Amotz, Ami, 1943- II. Polle, Jürgen E. W. III. Subba Rao, D. V.

QK569.P65A44 2008 579.8'32--dc22 2008048393

To

M. Avron, R.W. Butcher, A. Labbé, W. Lerche, N.P. Massjuk,
F.S. Milko, V.E. Semenenko, and E.C. Teodoresco
for their pioneering contributions on Dunaliella.

Preface

First described by Teodoresco in 1905, the unicellular green flagellate of the genus *Dunaliella* has been one of the most studied members of Chlorophyceae and is represented by 27 species, of which 23 are from salt water. Unique to *Dunaliella* cells is the absence of rigid cell walls, which characterize other unicellular green algae. Species of *Dunaliella* occur in freshwater, euryhaline habitats of all continents, oceans including the Dead Sea and even the salt lakes of the Antarctic. These extremophiles thrive in habitats with a wide range of salinity, pH, light intensity and temperature. The green vegetative cells of *Dunaliella salina* and *D. bardawil* under stress and light turn red due to over-accumulation of α and β-carotene, a feature that has biotechnological applications. Due to these unique features, interest in the fundamental physiological and biochemical research of *Dunaliella* has been increasing. Perhaps no other alga is studied as intensively as *Dunaliella* as a model for osmoregulation, pigment production and for commercial mass cultures.

The 21 chapters in this volume present a state-of-the art research in selected fields of *Dunaliella* including research in biochemistry, molecular biology and medical application. A glossary of specialized terms is appended. Each chapter is contributed by an expert or group of experts dedicated to increase our understanding of *Dunaliella*. All the chapters were reviewed internally by their colleagues, editors and external reviewers; this was followed by a final revision. Due to the range of subject matter, the 21 chapters contributed by 41 experts from 13 nations may vary in their format and style. Nevertheless, it is hoped this book provides a balanced multi-disciplinary communication and contributes to our understanding of this unique alga. We are most grateful to each of the contributors for their understanding, high level of professional and scholarly efforts, and for offering cordial and prompt cooperation in the preparation of this book. To our external reviewers we express our gratitude for their help with constructive comments in improving the content.

This book is addressed to postgraduate students and scientists as a summary of current thoughts on *Dunaliella*. We hope this book will serve as a stimulus

and catalyst for further research on *Dunaliella*; if so, as a contribution it will have served a useful purpose.

Subba Rao expresses special thanks to his wife Bala T. Durvasula for her infinite patience, and computer skills while keeping track of the correspondence between the three editors, widely-scattered contributors, and reviewers, and for excellent support in formatting the chapters and art work.

Acknowledgements

For Color figures/Photographs
We greatly appreciate the kind support of the Nikken Sohonsha Company and its late President Y. Tanaka, which made it possible to include color figures and photographs in this publication.

To Reviewers
The Editors reviewed the manuscripts first and submitted them to external reviewers. They extend their grateful thanks to the following conscientious reviewers who have selflessly contributed their time by providing constructive comments on the manuscripts and thus helped in producing this book. *Denotes reviewed more than one manuscript.

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1 History, Distribution, and Habitats of Algae of the Genus *Dunaliella* Teodoresco (Chlorophyceae)

Jürgen E. W. Polle[1], Duc Tran[1], and Ami Ben-Amotz[2]*

Abstract

Unicellular green algae of the genus *Dunaliella* TEOD. (Chlorophyta) have been used extensively as model organisms for various research areas and for mass culture. Since the early 19th century researchers have investigated the origin of the orange, purple, or pink color of water from salt lakes and salterns. Earlier it already was noticed that some kind of unicellular alga was causing the orange color of these hypersaline water bodies. This chapter reviews the history of the genus *Dunaliella* beginning with the earliest traceable records. In addition, worldwide distribution and habitats of algae of the genus *Dunaliella* are covered. As a specific example the Great Salt Lake in Utah, USA is described as a habitat of three recognized *Dunaliella* species.

Introduction

Unicellular green algae of the genus *Dunaliella* Teodoresco (Teodoresco 1905) were originally grouped in the class of Chlorophyceae, the order of *Volvocales*, and the family of *Polyblepharidacae*. Currently, order and family are still under debate. According to the National Center of Biotechnology Information of the National Institute of Health, algae of the genus *Dunaliella* belong to the class of *Chlorophyceae*, the order of *Chlamydomonadales*, and into the family of *Dunaliellaceae*. Chapter 2 by González et al. of this book discusses classification of the genus *Dunaliella* in more detail.

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Dunaliella species are mostly radially symmetrical, sometimes bilaterally symmetrical, flattened, dorsoventrally curved or slightly asymmetrical. The cell shape varies from ellipsoidal, ovoid, cylindrical, pyriform, or fusiform to almost spherical. Figure 1 shows a representative cell for *Dunaliella*. *Dunaliella* cells lack rigid cell walls, a feature distinguishing them from other unicellular green algae such as the genus *Chlamydomonas*. Nevertheless, *in vivo* cells of *Dunaliella* contain an almost invisible outer cell coat of variable thickness (Hamburger 1905, Teodoresco 1905, Figure 1). Already Teodoresco (1905) compared the cell coat as an envelope 'de nature peut-être protéïque' similar to the 'Hautschicht' of *Euglena*. Due to lack of a cell wall containing cellulose (Hamburger 1905), *Dunaliella* cells were often described as being 'naked' flagellates. Hamburger (1905) called the cell coat a 'Gallerthülle' (=mucilaginous coat) and stained it by use of Delafield Hämatoxylin or visualized it with Chinese ink. Later Labbé (1925) addressed the *Dunaliella* cell coat as a 'chlamyde' which could be stained by use of "blue-polychrome-orcéine" . Subsequent analysis (Oliveira et al. 1980, see also Ginzburg 1987) revealed that the cell coat could be stained by use of cationic dyes (Ruthenium Red, Alcian Blue) and it could be digested by trypsin or pronase. These results indicated that the mucilaginous cell coat of *Dunaliella* contains glycoproteins. The proposed makeup of the cell coat by glycoproteins is consistent with findings by Sadka et al. (1991) who described a high molecular weight glycoprotein of about 150 KDa existing in the outer layer surrounding *Dunaliella* cells. As the mucilaginous *Dunaliella* cell coat appears to be made up of glycoproteins, it is often referred to as a 'glycocalyx' (Borowitzka and Borowitzka 1988).

In general, vegetative cells of *Dunaliella* have two flagella of equal length of at least one body-length. If present, both flagella originate at the anterior end of cells.

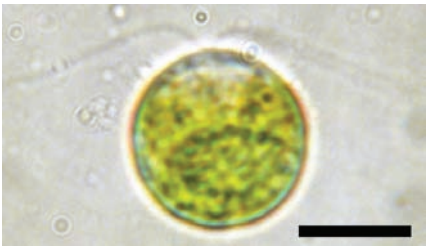

Figure 1: Photograph of an exemplary cell for algae of the genus *Dunaliella* (Magnification 360x). Both flagella originate at the anterior of the cell. The chloroplast fills the posterior region of the cell. The glycocalyx surrounding the cell is clearly visible as a white ring around the cell. The bar shown represents 10 μm.

Further, cells contain one single cup-shaped chloroplast with one central pyrenoid (Figure 2) surrounded by the storage product starch. Typical other organelles are anterior eyespots, anterior nucleus with nucleolus. Golgi bodies, and vacuoles. Cell size and shape may change within a given species depending on different environmental conditions varying in length between 2 to 28 µm and in width between 1 to 15 µm. Under non-optimal salt concentration, cell morphology may change to asexual sub-spherical, thick-walled cysts with bumpy surfaces that are often referred to as aplanospores (Borowitzka and Huisman 1993, Margulis et al. 1980). Nevertheless, the existence of aplanospores was questioned by Lerche (1937), who claimed that aplanospores are actually zygotes. Many species are extremely halotolerant and thrive on a wide range of salinities from seawater to salterns and evaporation ponds even in saturated salt solutions like the Dead Sea, Israel, and the Great Salt Lake, USA. Figure 2 shows exemplary cells of *D. viridis* and *D. salina* growing in concentrated brine solution. In addition, Figure 3 shows a cell of *Dunaliella spec.* that thrives in a concentrated brine solution containing visible salt crystals.

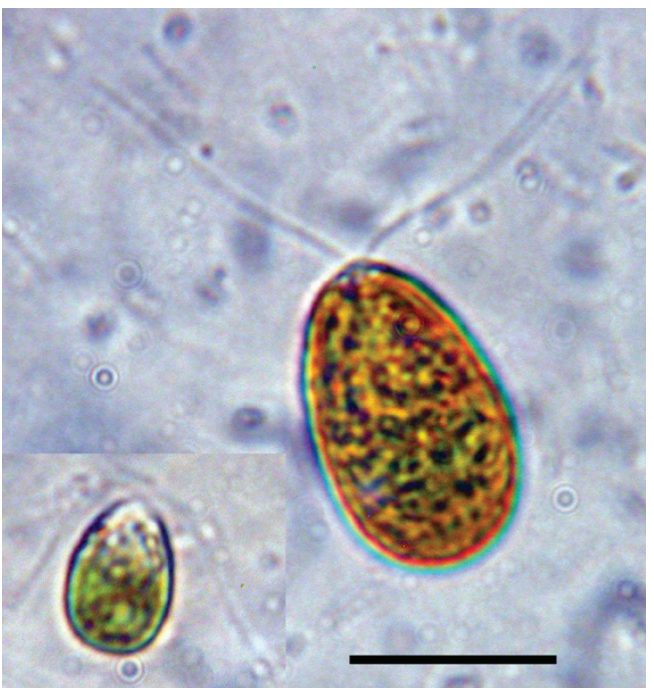

Figure 2: Shown are exemplary cells of *D. viridis* Teodoresco (lower left) and of *D. salina* Teodoresco (right side) grown in saturated brine solution (Magnification 360x). The bar represents 10 µm.

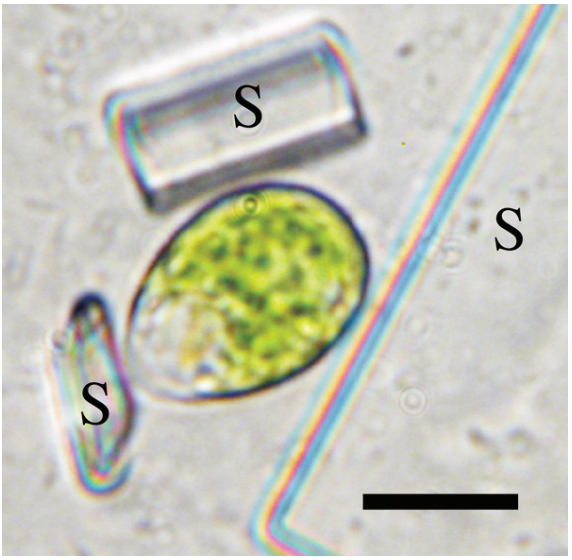

Figure 3: Photograph of a cell of *Dunaliella sp.* that is thriving in saturated brine solution at a magnification of 360x. S = salt crystals.

In past decades the species of *D. salina* Teodoresco and *D. tertiolecta* Butcher were widely used as model organisms for different research areas such as osmoregulation, carotenoid production, and photosynthesis under extreme conditions (Oren 2005). Currently, numerous strains of *Dunaliella* are deposited in different culture collections (http://www.dunaliella.org/dunabase/strains/strains.php), many with poor classification or erroneous characterization. In addition, various strains of *D. salina* are grown commercially in mass cultures for the production of natural β-carotene (Borowitzka and Borowitzka 1988, Ben-Amotz 2003).

History of the Genus *Dunaliella*

For centuries people wondered about orange-red lakes and red snow. Often the red color of waters reminded people of blood: "To the Moabites across the way, the water looked red—like blood" (Bible, 2 Kings 3.22). However, it was not until the microscope was discovered in the late 16th century that scientists could begin to reveal the mystery of red-colored waters and snow. A very recent review of the history of the species *Dunaliella* was presented by Oren (2005).

The earliest currently traceable scientific publication is from Turpin (1836, 1839) who recognized that the reddish coloration of salt lakes is caused by a

microscopic alga, which he named *Globularia kermesina*. At about the same time Dunal (1838) investigated the origin of the reddish color of salterns and described two microalgal species *Protococcus salinus* Dunal and *Haematococcus salinus* Dunal. Table 1 shows that following Dunal (1838) several scientists renamed the alga found in salt lakes and marine habitats multiple times. Finally, Teodoresco (1905) created the genus *Dunaliella* Teodoresco for the unicellular, halotolerant alga and recognized the type species *D. salina* Teodoresco (vegetative cells capable to turning red) (Teodoresco 1905, Hamburger 1905). Figure 4 shows cells of the type species *D. salina*. Vegetative cells are green under optimal environmental conditions and are 5.0 to 30.0 µm long and 2.5 to 21 µm wide. Depending on cell size and shape of the cell, the type species was divided into sub-species (Lerche 1937, Massjuk 1972, Preisig 1992). When exposed to environmental stress such as high salinity, vegetative cells turn orange (Figure 4B, 4C) due to over-accumulation of the pigment β-carotene (Loeblich 1982). Two decades later Labbé (1925) renamed the species *D. salina* Teod. into *D. kermesina* Turpin, because he recognized that Turpin had named this organism first. Labbé (1925) was not sure if an even earlier description of the species *Lepraria kermesina* by Wrangel from the year 1823 was also a synonym for *D. salina*. Unfortunately, the publication of Wrangel from 1823 cited by Labbé (1925) cannot be traced nor the accuracy verified, because of an incomplete citation by Labbé. Nevertheless, subsequent researchers did not accept the classification of the alga as *D. kermesina* Turpin and continued to use the name *D. salina* Teodoresco. Later, Ben-Amotz and Avron (1980a, b) isolated a new strain from the Bardawil Lagoon and named it *D. bardawil* Ben-Amotz et Avron. However, *D. bardawil* Ben-Amotz et Avron seems to fit the species characteristics of *D. salina* Teodoresco (Borowitzka and Borowitzka 1988, Ginzburg 1987, Gonzalez et al. 2001). At this stage classification of *D. bardawil* Ben-Amotz et Avron awaits reassessment through methods of modern molecular biology.

Table 1: Listed in chronological order by year of identification, names of species, and the author generating the taxon.

1 = *Dunaliella salina* variety according to species description of Teodoresco (1905)
2 = "*Chlamydomonas* I" Atkins, W.R. and Parke, M., 1951, J. Mar. Biol. Ass. U.K., 29, 609

Year	Species	Author
1836/39	*Globularia kermesina*	Turpin
1838	*Haematococcus salinus/ Protococcus salinus*	Dunal
1840	*Monas dunalii*	Joly
1841	*Diselmis dunalii*	Dujardin

(Table 1 Contd.)

(Table 1 Contd.)

1865	*Chlamydomonas dunalii*	Cohn
1872	*Protococcus salinus*	Geleznow
1886	*Sphaerella lacustris* var. *dunalii*	Hansgirg
1891	*Chlamydomonas dunalii*	Blanchard
1905	*Dunaliella salina*	Teodoresco
1906	*Dunaliella viridis*	Teodoresco
1925	*D. kermesina*[1]	Labbé
1935	*D. peircei*	Nicolai and Baas-Becking
1937	*1. D. parva* *2. D. media* *3. D. euchlora* *4. D. minuta*	Lerche
1938	*D. spec.* 1 *D. spec.* 2 *D. spec.* 3 *D. spec.* 4	Ruinen
1956 1959a	*D. bioculata*	Eddy Butcher
1959b	*1. D. tertiolecta*[2] *2. D. primolecta* *3. D. polymorpha* *4. D. quartolecta*	Butcher
1969	*D. turcomanica*	Massjuk
1971	*D. asymmetrica*	Massjuk
1973a	*1. D. maritima* *2. D. granulata*	Massjuk
1973b	*1. D. terricola* *2. D. gracilis* *3. D. ruineniana* *4. D. baas-beckingii* *5. D. minutissima* *6. D. carpatica* *7. D. jacobae*	Massjuk
1973	*D. pseudosalina*	Massjuk and Radcnenko
1980a,b	*D. bardawil*[1]	Avron and Ben-Amotz
1980	*D. marina*	Kombrink and Wöber

Soon after creation of the genus with the type species *D. salina*, Teodoresco recognized the second species *D. viridis* Teodoresco (vegetative cells always

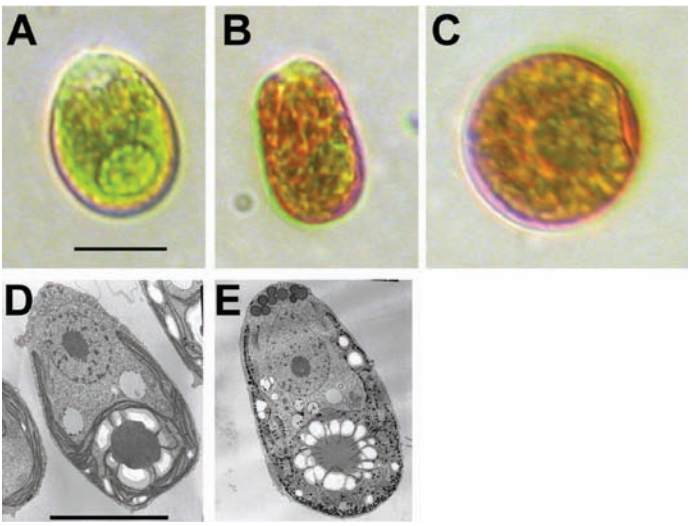

Figure 4: Photographs of cells of the type species *D. salina* Teodoresco at a magnification of 360x. A) Vegetative, motile, green cell grown at 1.5 M NaCl - the bar represents 10μm; B) Vegetative, motile, orange cell grown at 4.5 M NaCl; C) Orange cell grown at 4.5 M NaCl that is non-motile, either an aplanospore or a zygote; D) Electron micrograph of *Dunaliella bardawil, D. salina* variety according to species description of Teodoresco (1905), a green cell - The bar represents 10 μm; E) Electron micrograph of an orange cell. Photographs D & E reproduced by Dr. A. Ben-Amotz.

green) (Teodoresco 1906). Following generation of the genus *Dunaliella* and recognition of the two halotolerant species *D. salina* and *D. viridis*, many species were recognized during the last century, and characterized based on morphological and physiological features. Table 1 lists the year of identification, name of the species, authors, and provides references. Unfortunately, some of the species were only described once and not in great detail. Also, some of the *Dunaliella* species are not available from culture collections, or only one isolate exists among multiple collections.

Besides Teodoresco, major contributors to classification of species within the genus of *Dunaliella* were Lerche (1937), Butcher (1959a, b), and Massjuk (1969, 1971, 1973a, b, c, Massjuk and Radcnenko 1973). Following Labbé (1925), Lerche (1937) recognized great diversity within the species of *D. viridis*. Therefore, Lerche (1937) divided the species of *D. viridis* into several new species: *D. parva* Lerche, *D. peircei* Nicolai, *D. media* Lerche, *D. euchlora* Lerche, *D. minuta* Lerche. Later, Massjuk alone isolated and classified almost half of all currently known *Dunaliella* species. Massjuk's monograph contains very detailed information on

the morphology and physiology of various *Dunaliella* species (Massjuk 1973c). Unfortunately, this monograph is still only available in Russian and not well known. Overall, since Teodoresco (1905), 26 saltwater species have been described for the genus *Dunaliella*. Most of those species were grouped by Massjuk (1972) into four sections: *Tertiolectae* Massjuk, *Dunaliella* Massjuk, *Virides* Massjuk, and *Peirceinae* Massjuk.

In addition to saltwater species, several freshwater species were grouped in the genus of *Dunaliella* (Kalina 1965, Massjuk 1972, Pascher 1930, 1932, Pascher and Jehoda 1928, Skvortzov 1968). The freshwater species are placed into the subgenus *Pascheria*, whereas all saltwater species are placed into the subgenus *Dunaliella* (Massjuk 1972, Preisig 1992). Only five freshwater species are currently still classified as belonging to the genus *Dunaliella* (Preisig 1992). All five species are rare and only two of the species are available from culture collections. One of them is *D. acidophila* (Kalina) Massjuk (Melkonian and Preisig 1984) that was used specifically for investigation of pH homeostasis for several decades (Pick 1999). Classification of freshwater species to the genus *Dunaliella* is still questionable (Melkonian and Preisig 1984; Preisig 1992). For example, recent phylogenetic analysis revealed that the species of *Dunaliella lateralis* Pascher et Jahoda (Pascher and Jahoda 1928) should no longer be classified as belonging to the genus of *Dunaliella* (Gonzalez et al. 1999, Gonzalez et al. 2001).

Distribution and Habitats

Chlorophytes (green algae) of the genus *Dunaliella* can be found in freshwater and euryhaline waters of all continents and oceans (Lerche 1937, Massjuk 1972, Ginzburg 1987, Borowitzka and Borowitzka 1988, Preisig 1992). In addition, one species is found in acidic waters (*D. acidophila* Massjuk).

Reports of occurrence of euryhaline *Dunaliella* (*D. tertiolecta, D. primolecta*, *D. polymorpha*, and *D. quartolecta*) include marine saline waters in Europe and America. However, in marine phytoplankton *Dunaliella* species seem to play only a minor role, and their ecology is not well studied. In contrast, hypersaline species of *Dunaliella* (*D. viridis, D. parva*, *D. pseudosalina*, *D. salina*, and *D. spp.*) are commonly found from the Antarctic salt lakes to the salt lakes of Africa, America, Asia, Australia, and Europe (Table 2). In addition, hypersaline species of *Dunaliella* are present in evaporation ponds of salterns and salt marshes around the world. In hypersaline environments, species such as *D. salina, D. viridis,* and *D. parva* may play a major role in the ecosystems as primary photosynthetic producers of biomass. Often *Dunaliella* species give rise to blooms in salt lakes such as the Dead Sea (Oren and Shilo 1982, Oren 2005) or the Great Salt Lake (Post 1975). Typical examples of salt lakes and evaporation ponds with high brine concentrations are shown for North America in Figure 5. Although *Dunaliella*

species were found in numerous hypersaline environments, they do not exist in all hypersaline habitats (Ginzburg 1987). It appears that water chemistry plays a role in determining if *Dunaliella* species will be present in a hypersaline environment. According to Ginzburg (1987) the major limiting factors among the nutrients in salt lakes are nitrogen and phosphate.

Table 2: Habitats and distribution of some of the species of the genus *Dunaliella*.

Species	Habitats	Distribution
D. salina Teod.	Salt Lakes, Evaporation Ponds of Salterns, Hypersaline Salt Marshes or Lagoons	Africa, Americas, Asia, Australia, Europe
D. viridis Teod.	Salt Lakes, Salterns	Africa, Americas, Asia, Australia, Europe
D. parva Lerche	Salt Lakes	Asia, Europe
D. pseudosalina Massjuk	Salt Lakes, Hypersaline Salt Marshes or Lagoons	South America, Europe
D. tertiolecta Butcher	Brackish or Marine Waters	America, Europe
D. species	Salt Lakes	Antarctica

One example for a typical habitat for *Dunaliella* species is the Great Salt Lake which is located in the northern part of the state of Utah, USA. Pictures of the Lake are shown in Figure 5. It is one of the largest terminal lakes on earth. As the lake is relatively shallow, its size and salinity fluctuate depending on cycles of evaporation and surface freshwater inflow. For a long period the hydrogeochemistry of the Great Salt Lake was investigated (Spencer et al. 1985a, Spencer et al. 1985b, Stephens 1990). Since 1957 the Great Salt Lake has been divided by a causeway into the northern and southern basins. In general, the southern basin is less saline with 5-15% in comparison to the northern basin -also called Gunnison Bay- which has higher salinities of 15-28%. The phytoplankton in the Great Salt Lake has been the subject of various studies (Kirkpatrik 1934, Brock 1975, Post 1977, Post 1981, Stephens and Gillespie 1976, Van Auken and McNulty 1973). Among other microbes, at least three species of *Dunaliella* occur in the Great Salt Lake in significant numbers. *D. salina* exists mainly in the northern basin where the salinity is high (Brock 1975; Post 1981). In summer the entire northern basin may appear orange or pinkish in color due to blooms of *D. salina* and halobacteria (Figure 5). The reddish forms of *D. salina* contribute to an orange-ochre color wherease halobacteria are responsible for pink coloration. *D. viridis* exists in various parts of the lake, whereas a third unclassified *Dunaliella* species was found around Antelope Island (Brock 1975, Van Auken and McNulty 1973). The third as of yet unclassified species appears to belong to the section

Tertiolectae (Polle, unpublished results). Distribution of *Dunaliella* species within the lake is determined by salinity with *D. salina* being predominant at salinities around 25%. With an optimal salinity for growth of *D. salina* around 12% (Lerche 1937), its abundance at salinities of 25% is probably due to lack of competition by other algae and predation (Brock 1975). In contrast, the species *D. viridis* with an optimal salinity for growth of about 6% (Lerche 1937; Borowitzka et al. 1977) and the unidentified *Dunaliella* species are predominant at lower salinities in the southern basin. This example of the Great Salt Lake demonstrates that multiple species of *Dunaliella* co-exist in the same habitat.

Figure 5: Photographs showing views of the Great Salt Lake in Utah, USA. Green color of water is due to presence of a mixture of unicellular green algae including green forms of *Dunaliella* species. The pink color of water is caused by an abundance of halobacteria. A more orange water color is due to the unicellular green alga *Dunaliella salina*.

Summary

Unicellular green algae of the genus *Dunaliella* were studied since the early 19th century and numerous species were characterized and classified since then. Classification and ecology will be discussed in more detail in following chapters. In general, *Dunaliella* species are ubiquitous in saline environments and often multiple species occur in the same habitat. This wide distribution of species of the genus *Dunaliella* may be contributed to their tolerance to a wide range of salinities, light intensities, and temperatures as well as their ability to survive for many years among salt crystals (Ginzburg 1987).

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2 Taxonomy and Phylogeny of the Genus *Dunaliella*

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Abstract

In this chapter, the taxonomic position of the genus *Dunaliella* within the *Chlorophyta* is reviewed briefly, and some of the taxonomic difficulties arising from the use of morphological features at species level, evidenced by molecular studies, are analyzed. The genus *Dunaliella* comprises 28 recognized species separated in 2 subgenera (*Pascheria* and *Dunaliella*) with the subgenus *Dunaliella* being divided into 4 sections: *Tertiolectae*, *Dunaliella*, *Virids* and *Peirceinae*. All species belonging to the subgenus *Pascheria* were found in freshwater and there exist some cytological and molecular arguments suggesting that their taxa do not belong to the genus *Dunaliella* at all. Within the subgenus *Dunaliella,* sections are characterized by specific biochemical and physiological attributes while the species have been defined primarily by the morphological criteria. Consequently, the taxonomic validity of some of the 23 morphospecies traditionally described can be questioned. Further, the enormous intraspecific physiological and molecular variability of the hypersaline species *Dunaliella salina* Teodoresco is highlighted. Finally, some aspects on the major evolutionary trends and phylogeny within the subgenus *Dunaliella* are discussed.

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Taxonomic Position of the Genus *Dunaliella* within the Chlorophytes

When Teodoresco described the genus *Dunaliella* in 1905 he located it in the order *Volvocales* within the family *Polyblepharidaceae* (Teodoresco 1905), a position that was maintained by Lerche (1937) based on cell morphology, that is, flagellated unicells devoid of a cell wall. Much later Massjuk (1973) included the genus in the same order but in a new family, *Dunaliellaceae* erected by Christensen (1967) which in the original description includes "*single monad cells, with no membrane or lorica clothing them, known by their displaying the special nude flagellum of the Chlorophyceae*". Later, Ettl (1981) separated *Dunaliella* from the walled flagellates (i.e., order *Chlamydomonadales*, class *Chlamydophyceae*) into a newly erected order, *Dunaliellales* within the class *Chlorophyceae* (Ettl 1981, 1983). However, the order *Dunaliellales* sensu Ettl was polyphyletic (Nakayama et al. 1996). In consequence, three different views about the taxonomic position of *Dunaliella* currently exist which differ only in the order into which the family *Dunaliellaceae* sensu Christensen is placed:

	Alternative 1	Alternative 2	Alternative 3
Class:	*Chlorophyceae*		
Order:	*Chlamydomonadales*	*Dunaliellales*(sensu Melkonian 1990)	*Volvocales*
Family: *Dunaliellaceae*			
Genus: *Dunaliella*			

Alternative 1 is currently adopted by the National Center for Biotechnology Information (USA) and appears to be adopted by most researchers. Nevertheless, even though it is clear now that *Dunaliella* does not belong to the family *Polyblepharidaceae* - since most of those genera, if not all of them, have been transferred to the Class *Prasinophyceae* within the order *Polyblepharidales*, sensu Ettl 1983 - there is still confusion about the taxonomic position of *Dunaliella* at the ordinal and/or class level. An example of this is Oren (2005) who in his recent review paper '*A hundred years of Dunaliella research: 1905-2005'* still maintained the classical genus location (*Volvocales* and *Polyblepharidaceae*).

The ambiguity about the taxonomic position of the genus relates not only to the absence of a cell wall made up of cellulose, but presence of a 'Hautschicht' (Lerche 1937) consisting of a distinctive, probably glycoprotein cell cover (Ginzburg 1987, Massjuk 1973, Oliveira et al. 1980 in *D. tertiolecta*; Melkonian and Preisig 1984 in *D. salina*). It has been recognized by Melkonian and Preisig (1984) that *Dunaliella* is not just a wall-less equivalent of *Chlamydomonas.* The

authors mention that there are ultrastructural cell differences (i.e., the parabasal position of the dictyosomes, the system I fibres underlying two stranded microtubular roots, the presence of a prominent system II fibre [rhizoplast] and its association with mitochondria). On the other hand, Floyd (1978) and Mattox and Stewart (1984) cited similarities between both taxa: i.e., cell division – phycoplast and flagellar apparatus architecture - clockwise (CW) basal body configuration, respectively, and Chappell et al. (1989) suggested that *Dunaliella* lost the capacity to form a one-piece cell wall but retained the remnants of a phycoplast, and based on that presume a flagellated cell-walled ancestor for *Dunaliella* .

The advent of the molecular era and the relative simplicity in obtaining molecular data today together with the set of ultrastructural attributes available, will undoubtedly help to find the closest relatives to *Dunaliella* within the class *Chlorophyceae*. This aspect will be treated in the discussion of phylogeny.

How Many Species are Recognized within the Genus?

Since the original description of the type species *D. salina* (Teodoresco 1905) and later of *D. viridis* (Teodoresco 1906), many species from a wide range of habitats have been described (mostly by Lerche 1937, Butcher 1959a, b, and Massjuk 1973) (Table 1). The first monograph dedicated to *Dunaliella* was done by Labbé (1925) who focused on the species *D. salina*, which he renamed to *D. kermesina*, and *D. viridis*. A following monograph by Lerche (1937) realized that several different algae were combined under the name of *D. viridis* given by Teodoresco (1906). Consequently, Lerche (1937) erected several new species and eliminated *D. viridis* Teod. However, the species *D. viridis* was then again erected by Massjuk (1973). Lerche (1937) also focused on the life cycle and sexuality exhibited by some selected species (carotenogenic and non-carotenogenic taxa). She successfully described isogamy among five of the six species investigated by her (*D. salina*, *D. parva*, *D. peircei*, *D. euchlora*, and *D. minuta*); most of the species were homothallic. She found *D. salina* to be heterothallic with a weak tendency to homothallism. We have found repeatedly in different strains of *D. salina* that the species is homothallic (unpublished results). Lerche presented many valuable data on the physiology and biology of these organisms. She made the first attempt to use physiological (i.e. NaCl concentration for optimal growth, rate of cell division) and biological parameters (particularly, sexual reproduction) for species characterization. Later, Butcher (1959a) conducted taxonomic work based on cell morphology describing four new species (*D. tertiolecta*, *D. primolecta, D. quartolecta, D. polymorpha*) from the Bristish sea-coast. He used only cytological attributes such as pyrenoid shape, and number and location of granules within the cytoplasm, all criteria highly variable in green unicells.

Most of the above taxonomic studies were based solely on the morphological criteria (i.e., cell size and shape, cell symmetry, chloroplast shape, presence/absence

Table 1: List of the subgenera, sections and species of *Dunaliella* with their authorities. (*) Species included in this paper.

Subgenus *Pascheria*	
	1.- *D. acidophila* (KALINA 1965) MASSJUK 1971 2.- *D. flagellata* SKVORTZOV 1968 3.- **D. lateralis* PASCHER & JAHODA 1928 4.- *D. obliqua* (PASCHER 1930) MASSJUK 1973 5.- *D. paupera* PASCHER 1932
Subgenus *Dunaliella*	
Section *Tertiolectae* (oligo-euhaline)	
	6.- **D. maritima* MASSJUK 1973 7.- **D. polymorpha* BUTCHER 1959 8.- **D. primolecta* BUTCHER 1959 9.- **D. quartolecta* BUTCHER 1959 10.- **D. tertiolecta* BUTCHER 1959
Section *Dunaliella* (hyperhaline, acumulate carotenes)	
	11.- **D. parva* LERCHE 1937 12.- **D. pseudosalina* MASSJUK & RADCENKO 1973 13.- **D. salina* TEODORESCO 1905
Section *Virides* (hyperhaline, do not acumulate carotenes; cells radially symmetrical)	
	14.- *D. baas-beckingii* MASSJUK 1973 15.- **D. bioculata* BUTCHER 1959 16.- *D. carpatica* MASSJUK 1973 17.- *D. gracilis* MASSJUK 1973 18.- *D. granulata* MASSJUK 1973 19.- *D. media* LERCHE 1937 20.- **D. minuta* LERCHE 1937 21.- *D. minutissima* MASSJUK 1973 22.- *D. ruineniana* MASSJUK 1973 23.- **D. terricola* MASSJUK 1973 24.- **D. viridis* TEODORESCO 1906
Section *Peirceinae* (hyperhaline, do not accumulate carotenes; cells bilaterally symmetrical)	
	25.- *D. asymmetrica* MASSJUK 1973 26.- *D. jacobae* MASSJUK 1973 27.- **D. peircei* NICOLAI & BAAS BECKING 1935 28.- *D. turcomanica* MASSJUK 1969

of pyrenoid and eyespot, presence/absence of refractile granules). But it is widely recognized that cell shape is highly variable due to the absence of a rigid cell cover (Oliveira et al. 1980), and it is known that the cell size as well as the cell shape depend on external factors such as nutrients, pH, salt concentration, temperature,

and irradiation (Brown and Borowitzka 1979, Riisgard 1981, Ginzburg 1987). The presence/absence of eyespot and of refractile granules in the cells was also questioned by Preisig (1992). In contrast to the macro-morphological characteristics of numerous species which were studied by light microscopy, ultrastructural studies (Oliveira et al. 1980, Melkonian and Preisig 1984, Chardard 1987, Watanabe and Floyd 1989) were carried out on only 6 out of the 28 species of the genus. These ultrastructural studies described aspects of the flagellar root, cellular cover, chloroplast, pyrenoid, Golgi apparatus, and location of mitochondria within the cell. Most of these attributes appear useful at the genus level. Further, they appear to be very stable at the species level, at least in the studied taxa.

The most comprehensive work done on *Dunaliella* was that of Massjuk in 1973. She combined morphological and structural features (species level) with some physiological and biochemical attributes (section level) to divide the genus *Dunaliella* into two subgenera, *Pascheria* and *Dunaliella*.

The Subgenus *Pascheria*

The subgenus *Pascheria* (Table 1) comprises five species (*D. acidophila*, *D. flagellata*, *D. lateralis*, *D. obliqua*, and *D. paupera*), all of which occur in freshwater habitats. According to Melkonian and Preisig (1984) their inclusion within the genus *Dunaliella* is uncertain. They argued that *D. paupera*, *D. obliqua*, and *D. flagellata* lack a pyrenoid which is a characteristic attribute of the marine species. Moreover, both *D. paupera* and *D. obliqua* have unusual cell division, often producing unequal daughter cells. The fourth species, *D. acidophila* was originally described as *Spermatozopsis acidophila* Kalina (a name still in use by some scientists) from highly acidic habitats. The fifth freshwater species, *D. lateralis* is characterized by a distinctly lateral position of the pyrenoid, a feature not found in any of the marine species of *Dunaliella*. Apart from the distinctive features discussed above, all the freshwater species of the genus exhibit contractile vacuoles not found in their marine counterparts.

The results of ITS sequence data (González et al. 2001) agreed with the traditional view (based on cytological attributes) that *D. lateralis* is only distantly related to species included in the subgenus *Dunaliella*. To the best of our knowledge, there are no ITS or 18S rDNA sequences of the other species of the subgenus *Pascheria* available in the Genbank for comparative studies.

The Subgenus *Dunaliella*

Massjuk (1973) utilized physiological and biochemical criteria to group species in sections within the subgenus *Dunaliella*. She used the ability of various taxa

to grow optimally under different salt concentration ranges to separate them into two groups: Oligo-euhaline (2-4% NaCl) and hyperhaline (6-12% NaCl). Then, within the hyperhaline taxa she distinguished those that were capable of turning their cells yellow, brown, or brick-red in color (carotenoid accumulation) under extreme conditions from those taxa that kept their cells green.

Many authors have recognized the extraordinary capacity of taxa of *Dunaliella* to grow under a wide range of salt concentrations (e.g., Borowitzka et al. 1977, Ginzburg and Ginzburg 1981, Ginzburg 1987). Further, it was realized that this ability is related to the intrinsic characteristics of the strain and to its culture history (e.g., Brown and Borowitzka 1979, Latorella and Vadas 1973, Borowitzka et al. 1977). It was also documented that the oligo-euhaline taxa (sensu Massjuk 1973) were halotolerant (=grow better at low salinities of 0.4-4 % NaCl, but can tolerate up to 34% NaCl) whereas the hyperhaline taxa were halophilic (=grow better at higher salinities 6-12% NaCl). However, according to the above authors, the way in which halophilic species like *D. viridis* and halotolerant species like *D. tertiolecta* respond to different concentrations of salt is far from being understood. Although a physiological characteristic such as growth at specific salinities appears to be a good measure to delineate species, Ginzburg and Ginzburg (1981) showed the difficulty encountered in establishing optimal growth conditions for different halotolerant and halophilic species. This is due to interference between the environmental factors salinity, temperature, CO_2 level, and light. Further, these abiotic factors interact with others such as general ionic and nutrient composition of the medium and/or the natural environment (i.e., higher concentrations of NaCl can be tolerated under higher irradiances and higher concentrations of CO_2). In spite of such difficulties, Cifuentes et al. (2001) experimentally reaffirmed the validity of the physiological and biochemical attributes used by Massjuk (1973) to discriminate sections among the subgenus *Dunaliella*. In their work, Cifuentes et al. (2001) included two oligo-euhaline strains of *D. tertiolecta* (section *Tertiolectae*), and seven hyperhaline strains belonging to sections *Dunaliella*, *Virides*, and *Peircei*. They studied the effect of a wide range of salinities (1 to 30% NaCl w/v) on growth and pigment content in isolates that were acclimated or not acclimated to specific experimental conditions. The results revealed, as it was expected, two groups of strains: oligo-euhaline and hyperhaline. However, unexpectedly some strains of supposedly hyperhaline species (i.e., *D. parva* CCMP 362, *D. parva* CCAP 19/9, and *D. peircei* UTEX 2192) fell within the oligo-euhaline group (Figure 1 B, C, D). It was also found that even though *D. parva* UTEX 1983 fell within the hyperhaline group, it responded physiologically like *D. viridis* CONC-002 (high growth rate and optimal growth rates at intermediate salinities) (Figure 1 A, G). Based on these results, it was concluded that all the strains mentioned above (*D. parva* CCMP 362, *D. parva* CCAP 19/9, *D. peircei* UTEX 2192, as well as *D. parva* UTEX 1983) were probably misnamed when they were incorporated into the culture collections.

From the hyperhaline group, *D. pseudosalina* CONC-010 was the only strain whose culture turned light orange at late stationary phase of growth