

**PHYSIOLOGICAL ADAPTATION OF TWO UNICELLULAR
GREEN ALGAE TO pH STRESS**

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Abstract

Two marine algae, *Dunaliella parva* and *Chlorococcum submarinum* were selected to study the effect of pH stress on single celled algae. *D. parva* has been well characterised physiologically, but not with regards to pH stress. *C. submarinum* has not been so extensively studied, but is known to grow over a wide pH range from pH 4.5 to 10.5.

It was of prime importance that the algal cells were grown at the desired extreme values of external pH. Problems were encountered at high levels of pH but were overcome by growing the algae in a fermenter set up as a batch culture. This method of growth was used for both algae at extreme values of external pH and it allowed the accurate control of the media pH by the automatic addition of acid or alkali. pH 7.5 cells were grown in normal flask batch culture.

The cell number, cell volume, and chlorophyll content of both algae were determined over a wide range of pH values, showing that differences in external pH had significant effects on individual cells. Protein concentrations were measured and were shown to increase in pH 9.0 grown cells.

Determination of cell volume, internal pH and membrane potential have been carried out using radiolabelled isotopes for algae grown over a wide pH range. Cell volume was shown to increase at both acid and alkaline pH values. The internal pH of both algae was found to be at a more neutral pH than the external pH. For both *D. parva* and *C. submarinum*, the membrane potential increased with increasing external pH.

Enzyme activities in crude extracts were measured to establish the effects of external pH changes on metabolic pathways. The activity of these enzymes, taken from different organelles in the cell, was used to investigate the uniformity of internal pH.

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Contents

Chapter One : General Introduction

<u>1.1 Extreme Environments</u>	2
<u>1.2 Environmental Stress and Extremophiles</u>	2
1.2.1 Salt Stress	2
1.2.1.1 Halophiles	3
1.2.2 pH Stress	5
1.2.2.1 Acidophiles	8
1.2.2.2 Alkalophiles	9
1.2.3 Other	11
1.2.3.1 Temperature	11
1.2.3.2 Heavy Metals	12
<u>1.3 Dunaliella</u>	13
<u>1.4 Chlorococcum</u>	20
<u>1.5 Photosynthesis</u>	23
1.5.1 Oxygenic Photosynthesis	23
1.5.2 Inhibitors of Photosynthesis	26
1.5.2.1 Electron Transport Inhibitors	26
1.5.2.2 Energy Transport Inhibitors	27
1.5.2.3 Uncouplers	27
1.5.3 Other Inhibitors	27
<u>1.6 Aims of Project</u>	28

Chapter Two : Materials and Methods

<u>2.1 Provenance of Micro-organisms</u>	31
<u>2.2 Media and Growth Conditions</u>	31
2.2.1 <i>Chlorococcum submarinum</i>	32
2.2.1.1 Batch Culture	32

2.2.1.2 Fermenter Culture	32
2.2.2 <i>Dunaliella. parva</i>	33
2.2.2.1 Batch Culture	33
2.2.2.2 Fermenter Culture	35
2.2.3 Range of Buffers Use in Batch Culture	35
<u>2.4 Morphology of <i>C. subarinum</i></u>	36
<u>2.5 Chlorophyll Content</u>	36
2.5.1 <i>C. submarinum</i>	36
2.5.2 <i>D. parva</i>	37
<u>2.6 Protein Determination</u>	37
<u>2.7 Glycerol Determination</u>	38
<u>2.8 Photosynthesis and Respiration Measurements</u>	39
<u>2.9 Estimation of Cell Number</u>	42
<u>2.10 Estimation of Cell Volume by Micrometric</u>	
<u>Measurements</u>	43
<u>2.11 Silicone Oil Technique</u>	44
2.11.1 Determination of Cell Volume	45
2.11.2 Measurement of Membrane Potential	48
2.11.3 Determination of Internal pH	50
<u>2.12 Method for Intact Chloroplast Isolation</u>	51
2.12.1 Verification of Intact Chloroplasts	53
2.12.1.1 Microscope	53
2.12.1.2 NaH ¹⁴ C0 ₃ Fixation	53
2.12.2 Internal pH and ΔΨ Measurements of Intact	
Chloroplasts	54
<u>2.13 Enzyme Activities of <i>D. parva</i> and <i>C. submarinum</i></u>	
<u>at Different pH Values</u>	54
2.13.1 Preparation of Cell Free Extract	54
2.13.2 General Assay Conditions	55
2.13.3 Fumarase	56
2.13.4 Malate Dehydrogenase	57
2.13.5 Dihydroxyacetone (DHA) Reductase	58

2.13.6 Acid Phosphatase	59
<u>2.14 Statistics</u>	60

Chapter Three : pH Tolerance of *Dunaliella parva* 19/9 and *Chlorococcum submarinum*

<u>3.1 Introduction</u>	63
<u>3.2 Results and Discussion</u>	65
3.2.1 Maintenance of Medium pH using Buffers	65
3.2.2 Use of Fermenter to Maintain pH	68
3.2.3 Growth of <i>D. parva</i> and <i>C. submarinum</i> Over a Range of pH Values	69
3.2.4 Electron Micrographs of <i>D. parva</i> and <i>C. submarinum</i> Grown at pH 5.5, 7.5 and 9.0	72
3.2.5 Photosynthesis and Respiration Measurements	80
3.2.5.1 Cells Adapted to a Range of pH Values	80
3.2.5.2 Shock Experiments	83
3.2.5.3 Effect of Inhibitors	87
3.2.6 Cell Volume Determination in Different pH Media	94
3.2.6.1 Micrometric Measurements	94
3.2.6.2 Silicone Oil Measurements	97
3.2.7 Estimation of Cell Numbers at a Range of pH Values	101
3.2.8 Protein Determination in <i>D. parva</i> and <i>C. submarinum</i> Cells	103
3.2.8.1 Protein Determination per Alga Cell	105
3.2.8.2 Protein versus Cell Volume	106
3.2.9 Glycerol Determination in <i>D. parva</i> and <i>C. submarinum</i> cells	107
3.2.10 <i>C. submarinum</i> Growth after Resuspension in Fresh Media	110
<u>3.3 Conclusions</u>	111

Chapter Four : Bioenergetics of pH Tolerance in *Dunaliella* and *Chlorococcum*

<u>4.1 Introduction</u>	117
<u>4.2 Results and Discussion</u>	118
4.2.1 Isotope Uptake in Dead and Alive Algal Cells	118
4.2.2 Time Courses of Radiolabelled Isotopes	124
4.2.3 Effects of Unlabelled Dextran, TPP ⁺ , Methylamine and Benzoic Acid on <i>D. parva</i> and <i>C. submarinum</i>	125
4.2.4 Growth Conditions for Internal pH and Membrane Potential Experiments	127
4.2.5 Internal pH	129
4.2.6 Membrane Potential	132
4.2.7 Intact Chloroplasts	134
4.2.7.1 Verification of intact Chloroplasts	135
4.2.7.2 Internal pH	135
4.2.7.3 Membrane Potential	137
<u>4.3 Conclusions</u>	138

Chapter Five : Effect of Changing pH Levels on Intracellular Enzymes Extracted from *Chlorococcum* and *Dunaliella*

<u>5.1 Introduction</u>	143
<u>5.2 Results and Discussion</u>	144
5.2.1 Malate dehydrogenase	145
5.2.2 Fumarase	147
5.2.3 Dihydroxyacetone (DHA) reductase (also called Glycerol Dehydrogenase)	149
5.2.4 Glycerol Phosphate Dehydrogenase	151
5.2.5 Flagellar ATPase	152
5.2.6 Acid Phosphatase	153
<u>5.3 Conclusions</u>	155

<u>Chapter Six : General Discussion</u>	160
<u>Chapter Seven: References</u>	166
<u>Appendices</u>	184

Abbreviations

Abbreviations used in this thesis, not including elemental symbols and SI units are as follows:

a_i	concentration inside the cell
a_o	concentration outside the cell
ATP	Adenosine triphosphate
BSA	bovine serum albumin.
CAPS	3-[Cyclohexylamino]-1-propanesulfonic acid
CCCP	carbonylcyanide-m-chlorophenylhydrazone
Chl	chlorophyll
DHA	Dihydroxyacetone
DMSO	dimethylsulphoxide
dpm	disintegrations per minute
DTT	dithiothreitol
ECV	extracellular volume
g	gravitational force
HEPES	N-2-hydroxyethylpiperzine-N'-2-ethane sulphonic acid
ICV	intracellular volume
MES	2-[N-Morpholino] ethane-sulphonic acid
NAD	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (oxidised form)
OAA	oxaloacetic acid
OD	optical density
pH_i	internal pH
pH_o	external pH
Pi	inorganic phosphate

pK	is equal to the pH at which a compound is half dissociated
rpm	revolutions per minute
TCA	trichloroacetic acid
TPP ⁺	tetraphenyl-phosphonium cation
Tris	tris(hydroxymethyl) methylamine
v/v	volume per unit volume
w/v	weight per unit volume
Δ pH	transmembrane pH gradient.
$\Delta\psi$	transmembrane electrical potential

Introduction.

CHAPTER ONE

1.1 EXTREME ENVIRONMENTS

An extreme environment can be described as one in which some organisms are able to grow and reproduce whereas others cannot (Grant and Long, 1981). In virtually all examples of extreme environments it is found that as conditions become more and more demanding, micro-organisms predominate, and certain prokaryotic micro-organisms may exclusively occupy the most extreme niches (Grant and Long, 1981). Micro-organisms have been isolated from diverse environments such as hot deserts, frozen soils, sulphurous springs, alkaline lakes and extremely acid environments.

1.2 ENVIRONMENTAL STRESS AND EXTREMOPHILES

1.2.1 Salt Stress

Inland lakes in hot climates are the most extreme natural environments for salt tolerant microbes, for example the Dead Sea, Israel (Gilmour, 1990). The high rates of evaporation from these saline environments result from high temperatures and high light intensities. Highly saline lakes develop when evaporation exceeds the input of freshwater.

Extreme saline habitats are also found naturally in shoreline rockpools and estuaries (Gilmour, 1990). When the tide is out rockpools are exposed to evaporation and on a warm day salinity can increase several fold. The incoming tide then causes a rapid decrease in the salinity and thus rockpool organisms must be able to withstand sudden large fluctuations in salinity.

The activity of man also creates highly saline environments. In many parts of the world salt is produced by solar evaporation in ponds or salterns (Gilmour, 1990). Salterns are interconnected shallow ponds into which sea water is allowed and then retained while solar evaporation occurs (Cummings, 1991).

1.2.1.1 Halophiles

Tolerance to salt refers to the growth capability of the organism in a saline environment, and not to its ability simply to withstand high salt concentrations (Ben-Amotz and Avron, 1983). Organisms that are able to grow in conditions with or without high salinity levels are halotolerant organisms (0-1 M NaCl) and those that require high levels of salt for growth are usually called halophiles. Halophiles can either be moderate (0.1-4.5 M NaCl) or extreme (1.5-5.5 M NaCl) (Gilmour, 1990).

It has been shown that in halophiles, the internal concentration of NaCl does not equal the external concentration at high salinities (Gilmour, 1990). High concentrations of

sodium ions are toxic to all cells, therefore permeability to Na^+ is low and sometimes the ion is forced out of cells. The vast majority of halophiles use compatible solutes allowing the exclusion of the external NaCl whilst maintaining a favourable osmotic potential. Compatible solutes are solutes used inside the cell for adjustment of cytoplasmic water activity, they must be non-inhibitory to biochemical processes within the cell (Brown and Simpson, 1972; Brown, 1978). These compatible solutes are most commonly glycerol, proline and mannitol in algae (Gilmour, 1990), and glycine betaine and the novel cyclic amino acid ectoine in bacteria (Galinski, 1993).

Extremely halophilic archaeobacteria for example *Halobacterium* have been found in extreme but stable environments. They have become irreversibly adapted to high salt environments and have a requirement for a salt concentration of at least 2 M (Gilmour, 1990; Cummings, 1991). Halophilic archaeobacteria have high levels of intracellular cations which thus enable the cell to continue to function in the presence of high levels of salt. Significant levels of KCl and NaCl are found in these bacteria when extracellular salt concentrations are high (Cummings, 1991). The intracellular NaCl found is usually considerably less than the surrounding NaCl due to Na^+/H^+ antiports which restrict the back flow of Na^+ (Cummings, 1991). KCl is accumulated within the cell and is the compatible solute of these organisms (Cummings, 1991). Therefore, unlike all other halophiles, the halophilic archaeobacteria use an inorganic compatible solute (Galinski, 1983).

There are few algae that are able to grow across the full range of salt concentrations. Algae of the genus *Dunaliella*, extremely halophilic unicellular micro-algae, are able to thrive in media ranging from nearly freshwater (<0.1 M NaCl) to saturated salt solutions (>5 M NaCl) (Ben-Amotz and Avron, 1983). *Astereomonas*, a prasinophyte alga, has almost as wide a salinity range as *Dunaliella* with a salinity optimum of 0.5 - 4.5 M and thus it is considered to be moderately halophilic (Gilmour, 1990). *Chlamydomonas* sp. also fall in to the category of moderately halophilic as it has been reported to grow optimally in salinities up to 1.7 M NaCl (Gilmour, 1990). The red alga *Porphyridium* has a salinity optimum of 0.25 - 1.0 M and considered to be moderately halotrophic unlike the diatom *Cyclotella* which produces greatest growth at 0 - 0.5M NaCl and therefore termed halotolerant (Gilmour, 1990).

1.2.2 pH Stress

The pH of a solution is a measure of the concentration of H^+ . pH is defined as

$$pH = \log_{10} 1/[H^+] = -\log_{10} [H^+]$$

“It is the numeric value that indicates the relative acidity or alkalinity of a substance on a scale of 0 to 14, with the neutral point at 7. Acid solutions have pH values of lower than 7, and basic (alkaline) solutions have pH values greater than 7” (Miller, 1990).

Every aquatic organism has an external pH at which its rate of growth is maximal (Cano and Colome, 1986). Environmental pH directly affects micro-organisms and microbial enzymes (Atlas and Bartha, 1981). At an extremely high or an extremely low pH enzymes can be denatured and inactivated, and cell processes disrupted. It is therefore of prime importance that the internal pH of an organism never deviates from neutrality by more than about 2 pH units (Lim, 1989). Organisms that grow under extreme conditions of pH have to contend with effects other than high or low concentrations of H^+ or OH^- . These effects may result from metal ions being much more soluble at low pH or photosynthesis becoming affected by the availability of CO_2 (Borowitzka and Borowitzka, 1988; Grant and Long, 1981).

Natural acidic environments are more commonly found than alkaline environments (Sharp and Munster, 1986). Acidity develops in an aerobic oxidising environment by the oxidation of sulphides such as FeS_2 to sulphuric acid (Sharp and Munster, 1986). This process is accelerated by sulphur-oxidising bacteria and therefore extremely acid environments are commonly associated with areas of high sulphide concentration (Grant and Long, 1981). These habitats are dominated by such chemolithotrophic sulphur-oxidising bacteria, generally of the genus *Thiobacillus*, many of which have an optimum pH of between 2 and 4 and are unable to grow at neutrality (Krulwich and Guffanti, 1983). Moderately acidic natural environments between pH 3.0 and 4.0 are relatively common and include acid lakes, pine forest soils and acid bogs. More

extreme environments with pH values of less than pH 3.0 are often associated with human activity for example coal tips, drainage waters and mining effluents (Sharp and Munster, 1986; Prins *et al.*, 1990). The Dead Sea is an example of an acid saline lake enriched with cations. High levels of Mg^{2+} and Ca^{2+} are present (Oren, 1993). Fresh water flows in from the River Jordan but because there is no outlet the only form of water loss is through evaporation.

Alkalinity in terrestrial and aquatic environments may be generated by a number of factors, either naturally occurring or brought about by the activity of man (Grant and Tindall, 1986). Naturally occurring alkaline environments may arise by biological activity, such as ammonification, sulphate reduction and oxygenic photosynthesis and are relatively uncommon (Grant and Tindall, 1986; Sharp and Munster, 1986). In these instances the alkalinity may be localised and relatively short lived, or even diurnal (Grant and Tindall, 1986).

The most stable, and significant, naturally occurring alkaline environments are caused by a combination of geological and climatic conditions. Alkaline lakes and deserts so produced are geographically widely distributed. These are environments characterised by the presence of large amounts of sodium carbonate (or complexes of this salt), formed by evaporative concentration (Grant and Tindall, 1986). Formation of alkalinity in this way leads to the concentration of other salts (particularly NaCl), giving rise to alkaline saline environments. An example of a man made alkaline saline

environment is the Great Salt Lake in Utah in the United States which was divided in 1957 into a Northern and Southern Basin (Cummings, 1991). About 95% of the inflowing streams run into the Southern basin leaving the Northern basin with very little incoming water and high evaporation rates resulting in a virtually saturated brine.

Man-made alkaline environments are generally the result of waste from industries (Sharp and Munster, 1986). Commercial processes ranging from cement manufacture to the preparation of indigo dye give rise to environments with high pH (Grant and Tindall, 1986). Artificial environments above pH 10 include cement factory effluents which exceed pH 12 (Grant and Long, 1981).

1.2.2.1 Acidophiles

Acidophilic micro-organisms fall into two categories. Acid tolerant organisms are able to grow at pH values as low as 4.0, most are also able to grow at neutral pH. Obligate acidophiles grow at pH values of 2.0-4.0 but are unable to grow at neutral pH.

The enzymes and metabolic processes found in acidophiles function at a near neutral pH, therefore growth at low external pH requires the maintenance of a cytoplasmic environment at a relatively neutral pH. The ΔpH is actively maintained, this is not a passive response to control H^+ . At acid external pH the $\Delta\psi$ is reversed with the inside being positive. The maintenance of the near neutral internal pH is achieved by

pumping protons out of the cell or maintaining a cell surface barrier which is impermeable to the protons. In these acidophilic organisms internal pH can be primarily regulated by the respiratory systems in conjunction with the internal buffering capacity (Norris and Ingledew, 1992). Other systems such as an H⁺-ATPase or K⁺ cycling may also be involved (Norris and Ingledew, 1992). It has been noted that when compared to other bacteria, acidophilic bacteria show no differences in the composition of the cytoplasmic membrane (Norris and Ingledew, 1992).

1.2.2.2 Alkalophiles

Micro-organisms inhabiting alkaline environments may be divided into alkalophile and alkalotolerant types (Grant and Tindall, 1986). An alkalophile is an organism with an obligate requirement for alkaline growth conditions. It does not grow at neutral pH values and often grows optimally at pH values in excess of pH 10.0 (Krulwich *et al.*, 1990). An alkalotolerant (or alkalitrophic) organism is able to grow at alkaline pH, but grows optimally at lower pH values between 8.5 and 9.0 (Sharp and Munster, 1986). Alkalophiles include photosynthetic and non-photosynthetic bacteria, yeasts, fungi, diatoms and green algae (Kroll, 1990). In these organisms there are cellular structures outside the membrane such as the cell wall, outer membrane and flagella that are in direct contact with the external pH and thus must be able to function at high pH values (Kroll, 1990).

The intracellular pH of most alkalophilic bacteria appears to be between pH 7.0 and 9.0, although the external pH may be in excess of pH 10.0 (Krulwich *et al.*, 1990; Grant and Horikoshi, 1992). Antiport systems, which pump out cations and return protons to the cytoplasm, play an important role in the physiology of the alkalophilic bacteria (Krulwich *et al.*, 1990). They must combat the effects of respiration and external pH both of which tend to raise the intracellular pH (Sharp and Munster, 1986).

In all strains of alkalophiles when the external pH exceeds 9.5 the pH gradient is reversed, the inside of the cell becoming acid and the membrane potential ($\Delta\psi$) increasing with the increasing external pH. Alkalophiles have particular problems in generating energy by the chemiosmotic process since the normal pH gradient across the cell membrane is reversed (Grant and Horikoshi, 1992).

Micro-organisms growing at high pH exist in an environment essentially devoid of divalent cations such as Mg^{2+} and Ca^{2+} . These precipitate as carbonates and therefore organisms growing at high pH must have an efficient way of concentrating these metals.

1.2.3 Other

1.2.3.1 Temperature

The temperature at which an organism lives can place it into one of three groups for classification. Psychrophiles are found between -3 and 20 °C, mesophiles between 13 and 45 °C and thermophiles from 42 to 100 °C or more (Edwards, 1990). Organisms that are able to grow in excess of 90 °C and with an optimum of more than 65 °C are described as caldoactive (Sharp and Munster, 1986). Within these divisions there are subdivisions for species that do not fall easily into the major groups for example facultative thermophiles fall between mesophiles and thermophiles with a growth range of 30-60 °C.

Micro-organisms have been isolated from a wide range of environments with extremes of temperature. These include volcanic regions, solar-heated environments such as soil and ground litter, self-heating organic-rich materials such as compost heaps and seaweed piles, domestic and industrial hot water and cooling systems (Sharp and Munster, 1986). Low temperatures are a main influence in deep seas (>1000 m deep) and polar regions (Prieur, 1992). Some of the lowest optimum growth temperatures are those of the 'snow algae', which are found in snow covered habitats from alpine areas in the temperate zone to the Antarctic (Russell, 1992). These algae inhabit the upper 1 cm of snow and give it a red, green or yellow colouration (Russell, 1992).

1.2.3.2 Heavy metals

Many heavy metals, in inorganic or organic forms, are toxic to microbial life, although many are essential at low concentrations for normal growth (Gadd, 1992). Heavy metals compete with essential metals for active enzyme or membrane protein sites. They react with biologically active groups and thus may interrupt normal metabolic processes of algal cells (Visviki and Rachlin, 1991). Under conditions of metal pollution almost every microbial activity may be affected (Gadd, 1992). There are many factors that may effect the toxicity of the heavy metals, these include pH, temperature and salinity (Gadd, 1992; Visviki and Rachlin, 1991).

In general the abundances of metals in the environment are low and elevated areas occur naturally in specific locations such as deep sea vents and hot springs (Gadd, 1992). The activities of man have increased the metal distribution with for example brewery and distillery wastes, sewage treatment effluents and fungicides (Gadd, 1992).

1.3 *DUNALIELLA*

Dunaliella parva (Volvocales, Chlorophyceae)

The Chlorophyta (green algae) are primarily freshwater; only about 10% of the species in this group of algae are marine (Lee, 1989). In the marine environment, the green algae in the warmer tropical and semitropical waters tend to be similar everywhere in the world (Lee, 1989). This is not true of the Chlorophyta in the colder marine waters, because the waters of the Northern and Southern hemispheres have markedly different species (Lee, 1989).

Dunaliella species are widely distributed and may be found in freshwater e.g. *D. acidophila*, in estuarine rockpools e.g. *D. parva*, in hypersaline waters e.g. *D. salina* and in saline soil e.g. *D. terricola* (Borowitzka and Borowitzka, 1988). There is great variation in the cell shape of *Dunaliella* species, from ellipsoid, ovoid, cylindrical, pyriform and fusiform to almost spherical (Preisig, 1992). The cell shape in individual species can also vary depending on growth conditions especially if the cells are stressed. The morphology of *Dunaliella* cells is (nearly) uniform throughout the species. *Dunaliella* spp. lack a cell wall but have a mucilaginous cell coat on the external side of the membrane (Preisig, 1992). *D. parva* has cell dimensions of 9.9-16.0 x 4.0-10 μm (length x width) although there is great variation within the species in both cell length and width (Preisig, 1992).

Dunaliella parva has two flagella of equal length, giving the cell a rotational motion (Javor, 1989). Motility is essential for these cells as it enables them to control the amount of light received and aid in sexual reproduction in the mating of gametes (Marano, 1992). A single, cup-shaped chloroplast, occupies most of the cell body (Preisig, 1992; Borowitzka and Borowitzka, 1988). In marine and halophilic species a central pyrenoid is found within the chloroplast (Borowitzka and Borowitzka, 1988).

Reproduction in *Dunaliella* spp. may be vegetative by lengthwise division in the motile state (Preisig, 1992). Palmelloid stages, where a mass of nonmotile cells are encased in mucus, may be formed under certain conditions (Preisig, 1992). Rarely, under extreme conditions, such as drying up of the environment, cysts with thick rough walls may be formed (Borowitzka and Borowitzka, 1988). Sexual reproduction is by isogamy. The gametes have the same size and same structural features as growing cells of the same species (Preisig, 1992). This procedure is similar to that found in *Chlamydomonas* (Harris, 1989). *D. parva* is morphologically very similar to *Chlamydomonas*, differing from this genus mainly in the absence of a cell wall.

Dunaliella spp. have chlorophylls *a* and *b* and form starch within the chloroplast (Lee, 1989). A range of carotenoids and xanthophylls are also found within the cells (Borowitzka and Borowitzka, 1988). *D. salina* and *D. baradawil* cells synthesise large amounts of β -carotene in response to salt or other stresses. β -carotene rich

Dunaliella strains are widely distributed in salt water bodies that contain a high percentage of salt (Borowitzka and Borowitzka, 1988; Shaish *et al.*, 1992). The carotenoid β -carotene is of great interest in biotechnology as a health food, for food colouring and in animal feed (Ben-Amotz and Avron, 1990). *Dunaliella* spp. are farmed in large open air hypersaline ponds in several countries throughout the world to harvest β -carotene. These large scale bioreactors can encounter many problems such as mixing and light penetration. Contamination is not normally a problem with these particular bioreactors as the salt concentration in the growth medium is so high that most contaminants are unable to survive (Ben-Amotz and Shaish, 1992).

Dunaliella spp. have several requirements for growth. Inorganic carbon is essential for survival. This is probably the most limiting growth factor for extremely halophilic *D. salina* cells, because as the salinity of the growth medium increases the solubility of inorganic carbon decreases (Borowitzka and Borowitzka, 1988). Nitrogen is also a major requirement. This is taken up in the form of nitrate or nitrite which causes alkalisation of the medium, or as ammonia which acidifies the medium (Borowitzka and Borowitzka, 1988). The marine and halophilic species of *Dunaliella* have a specific requirement for sodium (Borowitzka and Borowitzka, 1988). Other elements such as phosphorus, magnesium, calcium etc. are required in moderation, but additional vitamins are not required (Borowitzka and Borowitzka, 1988).

Dunaliella spp. are primarily noted for their halotolerant properties. In response to changes in external sodium chloride concentrations these algae are able to accumulate or degrade large amounts of intracellular glycerol for osmotic balancing (Ben-Amotz and Avron, 1983). The mechanism of adaptation to increased salinity appears to be straight forward. Following hyperosmotic shock water flows out of the cell causing a decrease in cell volume. The decrease in volume triggers the production of glycerol from starch or from photosynthetically fixed CO₂. If the salinity increase is large, photosynthesis is inhibited and all the glycerol is produced from starch (Gilmour, 1990). Glycerol production is initiated in the chloroplast where starch is broken down to dihydroxyacetone phosphate via glucose and fructose (Avron, 1992). Over time the cell volume recovers to approximately the volume before the salt shock and growth recommences with a higher concentration of glycerol inside the cells. Exactly the opposite procedure occurs when the cells are subjected to salinity decreases, glycerol is converted back to starch or if the salinity decrease is large glycerol is excreted into the medium (Gilmour, 1990).

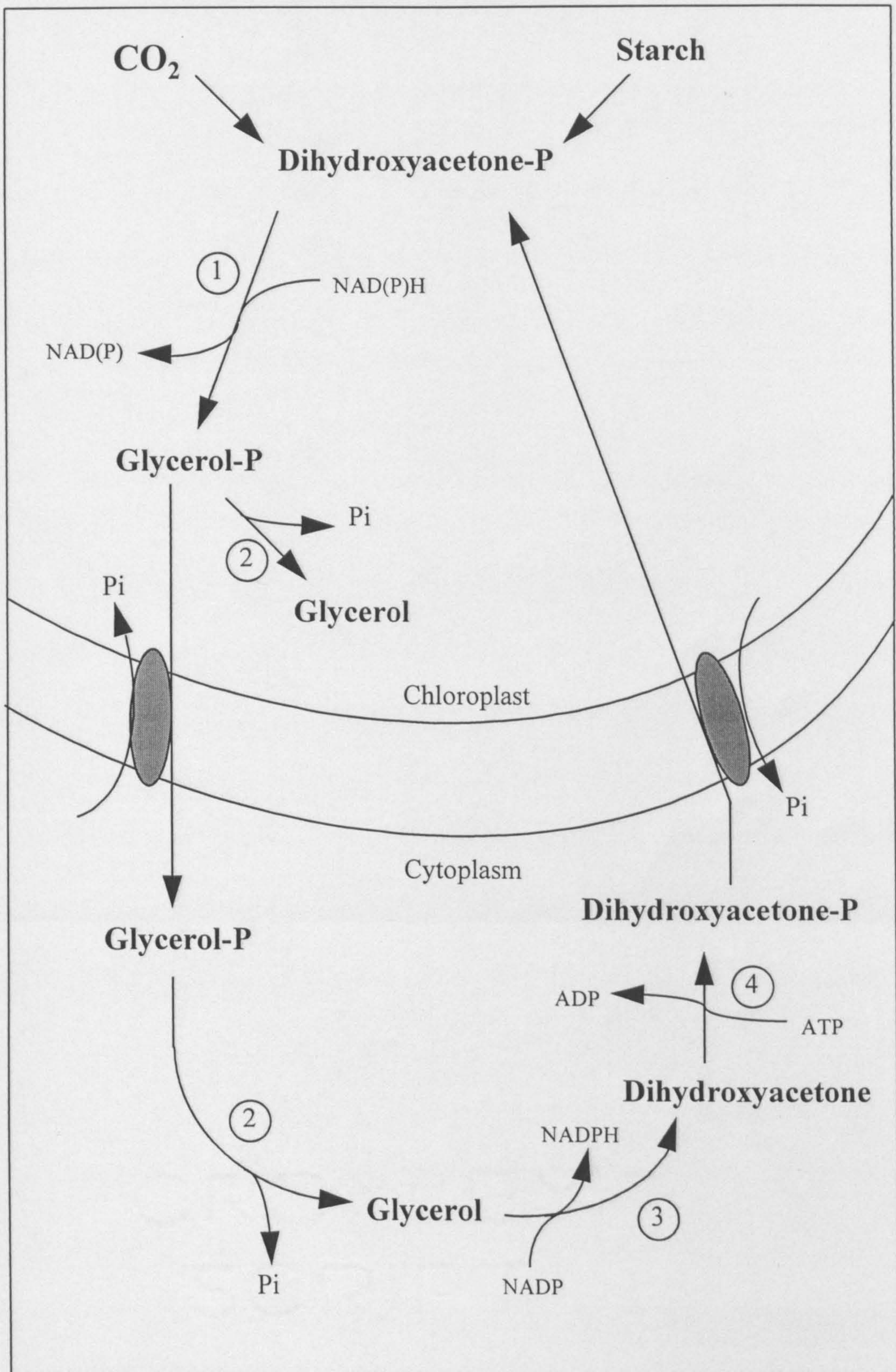
The production of dihydroxyacetone phosphate is the starting point of the glycerol cycle in which glycerol accumulates in the chloroplast and the cytoplasm (Figure 1.1). The initial conversion of dihydroxyacetone phosphate to glycerol phosphate is catalysed by a dehydrogenase enzyme found only in the stroma of the chloroplast. The phosphatase reaction which catalyses the formation of glycerol takes place in the chloroplast and the cytoplasm and is an irreversible reaction. To allow glycerol

production in the cytoplasm, glycerol phosphate is transported out of the chloroplast via an inorganic phosphate (Pi) transporter (Figure 1.1). Due to the irreversible nature of the phosphatase reaction, glycerol is converted back to dihydroxyacetone phosphate by two enzymes dihydroxyacetone reductase and dihydroxyacetone kinase, which are both found in the cytoplasm. The dihydroxyacetone phosphate is then transported back into the chloroplast by a Pi transporter and is subsequently converted back to starch (Figure 1.1).

Figure 1.1: The Glycerol Cycle (adapted from Avron, 1992).

- ① **Glycerol phosphate dehydrogenase** - present in chloroplasts only (reversible reaction),
 - ② **Glycerol phosphate phosphatase** - present in both chloroplast and cytoplasm (irreversible reaction),
 - ③ **Dihydroxyacetone reductase** - present in cytoplasm only (reversible reaction),
 - ④ **Dihydroxyacetone kinase** - present in cytoplasm only (irreversible reaction).
-

Figure 1.1 : The Glycerol Cycle



The trigger for glycerol synthesis or degradation is the change in cell volume, however until recently the mechanism that senses the cell volume was unclear. Then in 1995 Zelazny and co-workers suggested that sterols in the plasma membrane may be involved in the activation of the synthesis of glycerol through a plasma membrane sensor. Sterols have a important effect on the structure and physical properties of membranes as well as lipid organisation and it is thought that a hyperosmotic shock may activate the sensor and bring order to the phospholipids and sterols in the membrane, thus triggering glycerol synthesis (Zelazny *et al.*, 1995).

Over time the cell volume recovers to approximately the volume before the salt shock and growth recommences with a higher concentration of glycerol inside the cells. Exactly the opposite procedure occurs when the cells are subjected to salinity decrease (Gilmour, 1990). Both synthesis and breakdown of glycerol require ATP, but protein synthesis is not a requirement (Borowitzka and Borowitzka, 1988).

Dunaliella spp. have a wide temperature tolerance, cells may remain viable over a range of temperatures from -3 °C to 40 °C, but no one species can survive the full temperature range (Borowitzka and Borowitzka, 1988). Cells adapted to high salinities are more temperature resistant, this could be the result of the additional glycerol content in the cells resulting from the salt stress (Borowitzka and Borowitzka, 1988).

Dunaliella spp. can tolerate pH levels from pH 1, where *D. acidophila* is able to grow, to pH 11 which can support growth of *D. salina*, but no one species can survive the full pH range (Borowitzka and Borowitzka, 1988). The optimal pH for photosynthesis is generally low, and most individual *Dunaliella* species can tolerate a range of pH values from pH 5 to pH 9 (Borowitzka and Borowitzka, 1988).

D. acidophila (formerly *Spermatozopsis acidophila*) produces optimal growth at pH 1. This alga exhibits a positive membrane and surface potential and it is thought that these play a key role in the acid resistance of this alga (Hirsch *et al.*, 1993). These positive potentials act as expelling forces for external protons and thus help maintain a near neutral cytoplasmic pH (Carandang *et al.*, 1992). See section 4.1 for more details of the bioenergetics of pH tolerance.

1.4 *CHLOROCOCCUM*

Chlorococcum submarinum (Chlorococcales, Chlorophyceae)

Chlorococcum spp. have been isolated from various habitats through out the world. *C. fissum* was isolated from silt in a lake in central Asia, *C. scabellum* from warm desert soil in south-western America and *C. lacustre* and *C. oleofaciens* were isolated from peat soil in a deciduous forest in north-eastern America respectively (Archibald, 1988). *C. submarinum* can be found in marine coastal habitats in the United Kingdom. First

isolated by Russell and Mott in 1976 and reported to the annual meeting of the British Phycological Society. It has been morphologically described in detail by Blackwell *et al.* (1991). In the vegetative growing state, *C. submarinum* cells are spherical in shape with diameters of 10-15 μm and a thick cell wall up to 1 μm thick. During the stationary phase the cells are more oval in shape with dimensions of 5-7 x 8-11 μm with a thinner cell wall (up to 0.5 μm). *Chlorococcum* has a single nucleus and a cup shaped chloroplast with a distinct pyrenoid body in the thickened basal region (Blackwell *et al.*, 1991).

Asexual reproduction involves the division of the protoplasm to produce zoospores and aplanospores. The zoospores are 3 μm wide and 8 μm long with two flagella of equal length. Sexual reproduction is by fusion of isogametes are indistinguishable from zoospores (Blackwell *et al.*, 1991).

C. submarinum produces maximal growth in the salinity range 0.1 to 0.5 M NaCl with little variation in growth rates across this range. Studies have established that *C. submarinum* can be grown at salinities up to 2 M NaCl, albeit at a reduced rate (Blackwell and Gilmour, 1991a). Blackwell and Gilmour (1991b) established that the intracellular glycerol content of *C. submarinum* cells varies with the external salinity. *C. submarinum* produces a higher lipid content when placed in stressed conditions, but β -carotene does not seem to be involved (Blackwell, 1990).

This alga has been found to have exceptional tolerance to variation in external pH (Blackwell and Gilmour, 1991a). Growth is unaffected by pH across the range 4.5 to 10.5 but is significantly lower at pH 3 and very poor at pH 2 (Blackwell and Gilmour, 1991a). Schnackenberg *et al.* (1996) discovered that resuspending *C. littorale* in pH 11 affected the PS II system. The high pH was found to inhibit the release of O₂ from H₂O in PS II.

The upper pH limit is unknown because no suitable buffering agent has been found which could hold the pH steady above pH 10.5 for growth in batch cultures (Blackwell and Gilmour, 1991a). The mechanism(s) used by *C. submarinum* to tolerate extremes of pH is unknown.

There has only been a limited amount of research done on *Chlorococcum* spp. Most of the recent published research has originated from Japan. *Chlorococcum* strain HS-101 has been of great interest in Japan as methanol extracts from this algae strongly inhibit the growth of a strain of *Staphylococcus*. *S. aureus*, which is resistant to many antibiotics and can cause diarrhoea, fever and sometimes death in hospital patients (Ohta *et al.*, 1993, 1994, 1995). It was established that antibiotic activity was due to unsaturated fatty acids found in *Chlorococcum* HS - 101, and other photosynthetic algae (Ohta *et al.*, 1995).

1.5 Photosynthesis

Both *Dunaliella* and *Chlorococcum* carry out higher plant type oxygenic photosynthesis, with two photosystems and the evolution of oxygen. The products of the light-dependent reactions of photosynthesis are ATP and NADPH, which are then used in the “dark” reactions to fix CO₂ into organic compounds (Lawlor, 1987a,b).

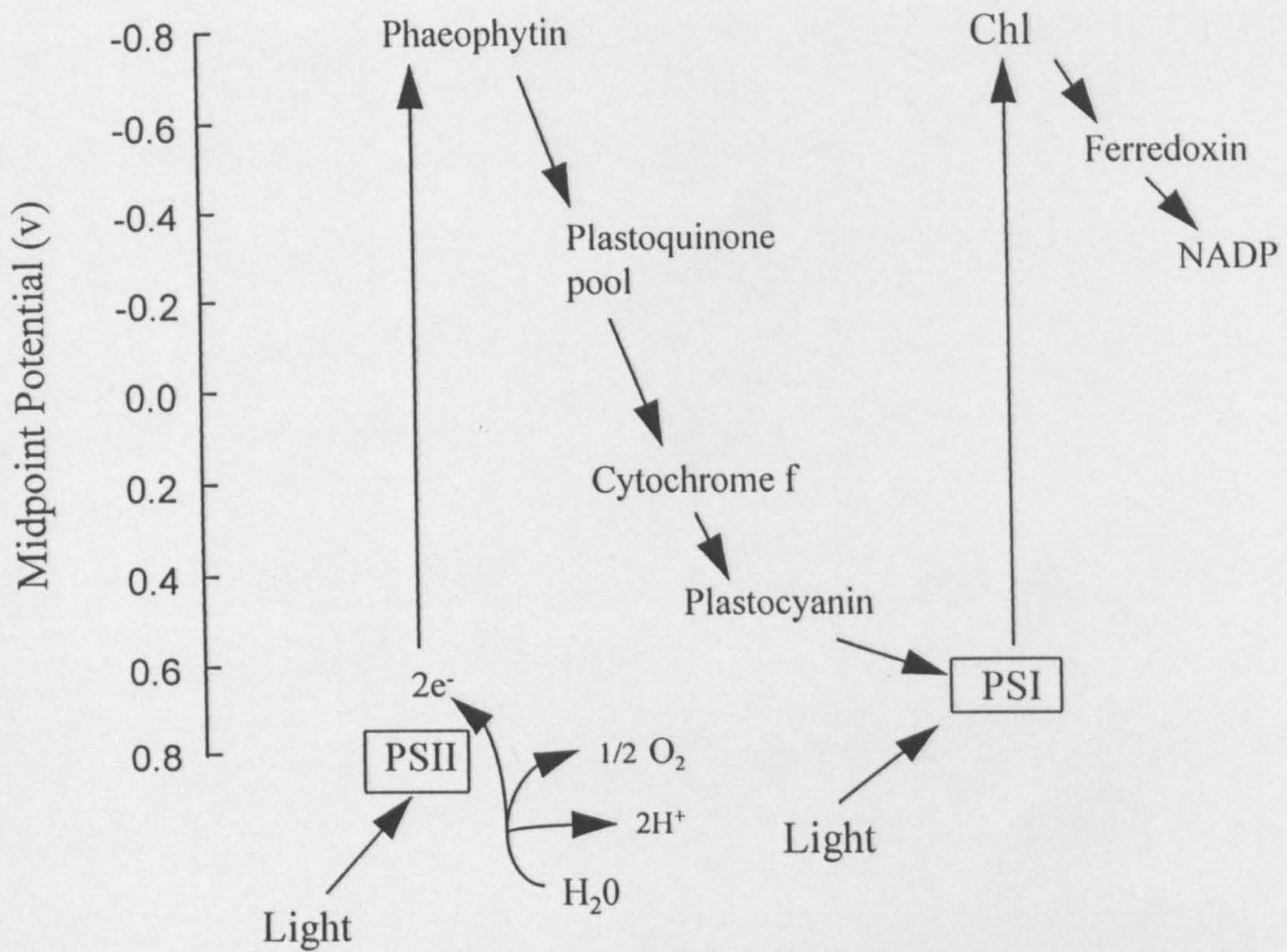
1.5.1 Oxygenic Photosynthesis

In plants and algae oxygenic photosynthesis takes place in the organelle called the chloroplast. Within the chloroplasts are sack-like structures called thylakoids which can be stacked into grana. Once light is trapped by photosynthetic pigments, the light energy is transferred to reaction centres of photosystem I and II (PSI and PSII), which are found in the thylakoid membranes (Lawlor, 1987a). The light energy is then used to drive electrons to more negative redox potentials and this scheme of non-cyclic photosynthetic electron transport is shown in Figure 1.2(a,b). Following absorption of a light quantum and transfer of energy to the reaction centre, PSII generates a sufficiently negative redox potential to pull electrons from water and eject them up the redox potential scale to the primary acceptor, pheophytin. Electrons then pass down the redox potential scale through a series of intermediates until at PSI another quantum of light pushes them to a very negative redox potential. Thus the two photosystems working together allow electrons to reach a negative enough redox potential to directly reduce NADP to NADPH (Lawlor, 1987a).

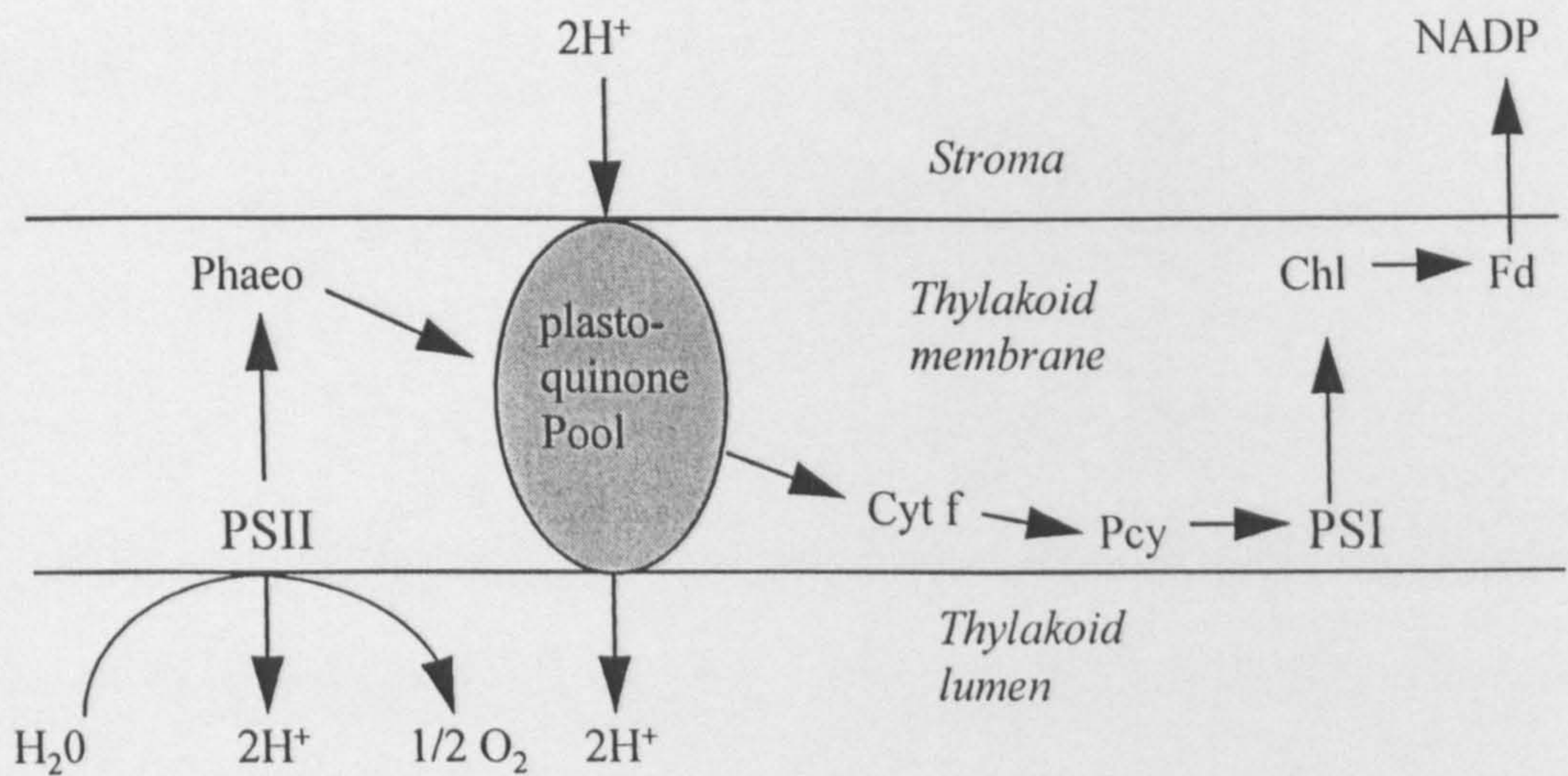
To understand how ATP is produced in this process, the spatial orientation of the electron transport components in the membrane needs to be examined (Figure 1.2b). This reveals that the water splitting reaction of PSII takes place at the inner side of the thylakoid membrane and the H^+ liberated in this reaction move into the thylakoid lumen (Lawlor, 1987b). In addition when electrons pass from the plastoquinone pool (H^+ plus electron carrier) to the cytochrome b/f complex this also takes place at the lumen side of the thylakoid membrane and the H^+ are again released into the lumen (Figure 1.2b).

Figure 1.2: (a) The 'Z-scheme' of photosynthetic electron transport and (b) a schematic diagram to illustrate the orientation of electron transport components in the thylakoid membrane with respect to proton translocation (adapted from Lawlor, 1987a,b).

Figure 1.2 (a) The 'Z' scheme of photosynthetic electron transport



(b) Orientation of electron transport chain in the thylakoid membrane



Therefore, electron transport transport from H_2O to NADP sets up a gradient of H^+ across the thylakoid membrane. Some electrons can cycle around PSI and this increases the H^+ gradient across the membrane, but unlike non-cyclic electron transport from H_2O to NADP, no NADPH is produced (Lawlor, 1987a). The gradient of H^+ across the thylakoid membrane is called the proton motive force (pmf) and it is made up of two components: a pH difference (ΔpH acid inside) and a membrane potential ($\Delta\Psi$, inside positive). The pmf is the driving force that pushes H^+ through F_1/F_0 ATPases in the thylakoid membrane to generate ATP (Figure 1.2b).

1.5.2 Inhibitors of Photosynthesis

Inhibitor studies have been very useful in discovering the photosynthetic mechanisms described in section 1.5.1. Selective inhibition of different parts of the photosynthetic pathways confirm their roles in the process and selective inhibitors can be used to see what role photosynthesis plays in a cell's response to environmental stress. The major groups of inhibitors are briefly described below.

1.5.2.1 Electron Transport Inhibitors

Inhibitors of this class interfere with photosynthetic electron transport e.g. DCMU which acts between pheophytin and the plastoquinone pool. It blocks non-cyclic electron transport, but cyclic electron transport is unaffected. Therefore, DCMU can be used to study the role of cyclic electron transport in photosynthetic cell metabolism (Nicholls and Ferguson, 1992).

1.5.2.2 Energy Transfer Inhibitors

These compounds act directly on ATPases by preventing passage of H^+ through the ATPase e.g. DCCD (Nicholls and Ferguson, 1992). This stops ATP synthesis and eventually stops electron transport due to “back pressure” from H^+ building up within the thylakoid.

1.5.2.3. Uncouplers

Uncouplers (e.g. CCCP) transport H^+ down a concentration gradient. They abolish both ΔpH and $\Delta\Psi$ and therefore uncouple electron transport from ATP production. In the short term uncouplers cause electron transport to proceed at its maximal rate, since there is no “back pressure” from H^+ inside the thylakoids (Nicholls and Ferguson, 1992). Uncoupling results in a short term increase in oxygen evolution.

1.5.3 Other Inhibitors

Ionophores are a class of compounds which induce membranes to become more permeable to ions. CCCP (section 1.5.2.3) is one example and monensin is another. Monensin allows Na^+ ions to pass through membranes and is often used to affect the function of the Na^+/H^+ antiporter (Dibrov *et al.*, 1986).

1.6 Aim of Project

The aim of this study was to investigate the mechanisms by which tolerance to extreme environmental conditions is accomplished. This was examined by growing the algae over the pH range 5.5 to 9.0.

The following areas will be covered in this thesis for both *Dunaliella parva* and *Chlorococcum submarinum*:

Cell characterisation in different pH values (Chapter 3).

Bioenergetics of pH tolerance (Chapter 4).

Effect of changing pH on intracellular enzymes (Chapter 5).

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Materials and Methods.

CHAPTER TWO

2.1 Provenance of Micro-organisms

Dunaliella parva (strain 19/9) and *Chlorococcum submarinum* (strain 213/10) were obtained from the Culture Centre of Algae and Protozoa, Scottish Marine Biological Association, Oban, UK.

2.2 Media and Growth Conditions

All media were prepared using distilled water and unless stated otherwise sterility was achieved by autoclaving for 20 min at 121°C (15 lbs in⁻¹).

The composition of each medium is shown in Appendix A and B. The two principal basal media prepared were Woods Hole MBL medium (Nichols, 1973) (*Chlorococcum submarinum*) and *Dunaliella* artificial seawater medium (Hejibagheri *et al.*, 1986) (*Dunaliella parva*). Unless otherwise stated *C. submarinum* was grown at 0.25 M NaCl, (20 mM Tris pH 7.5 buffer) and *D. parva* was grown at 1.5 M NaCl (100 mM Tris pH 7.5 buffer).

For both algae, two sets of growth conditions were used: batch culture and fermenter culture.

2.2.1 *Chlorococcum submarinum*

2.2.1.1 Batch Culture

For liquid cultures the *C. submarinum* was grown in cotton wool plugged sterile 250 ml conical flasks containing 100 ml Woods Hole MBL medium. The cultures were incubated in a VSL (Vindon Scientific Limited, Oldham, England) incubator at a constant $20 \pm 1.5^\circ\text{C}$, illuminated continuously with an intensity of 50-60 $\mu\text{mol s}^{-1} \text{m}^{-2}$ and shaken at 100 rpm. The inoculum was usually 5 ml of a 14 day old culture and cells were used for experiments after 10-14 days.

2.2.1.2 Fermenter Culture

C. submarinum was also grown using a LH 500 series fermenter (LH Fermentation Ltd, Stoke Poges, UK). When the alga was to be grown in the fermenter normal Woods Hole medium was employed, initially set at pH 7.5. Temperature was maintained at 20°C with the combination of a heater and a cold finger and the cells were constantly mixed at 500 rpm by an agitator. Air was bubbled through the culture at $500 \text{ ml minute}^{-1}$. Fermenter culture was used to allow accurate control of the medium pH. This was achieved by the automatic addition of sterile 1 M NaOH or 1 M HCl, when the pH deviated from pre-set limits.

800 ml of medium was placed in the culture vessel and medium was also placed in a 1 litre side-arm flask which was connected via tubing to the reaction vessel. This

allowed for fresh medium to be added during growth in the fermenter. The reaction vessel, side-arm flask, acid and alkali reservoirs and waste reservoir (Figure 2.1) were autoclaved at 121 °C (15 lbs in⁻¹) for 15 minutes.

An inoculum was added to the growth vessel, to give a concentration of approximately 0.33 µg Chl ml⁻¹. Once inoculated the algae were allowed to grow for about a week before the pH of the medium was altered.

2.2.2 Dunaliella parva

2.2.2.1 Batch Culture

For liquid cultures *D. parva* was grown in cotton wool plugged sterile 250 ml conical flasks containing 100 ml of *Dunaliella* artificial seawater medium. The cultures were incubated under constant illumination (20-25 µmol quanta m⁻² s⁻¹) at 25 °C with occasional manual shaking. The inoculum was usually 5 ml of a 14-21 day old culture and harvesting normally occurred after 10-14 days of growth.

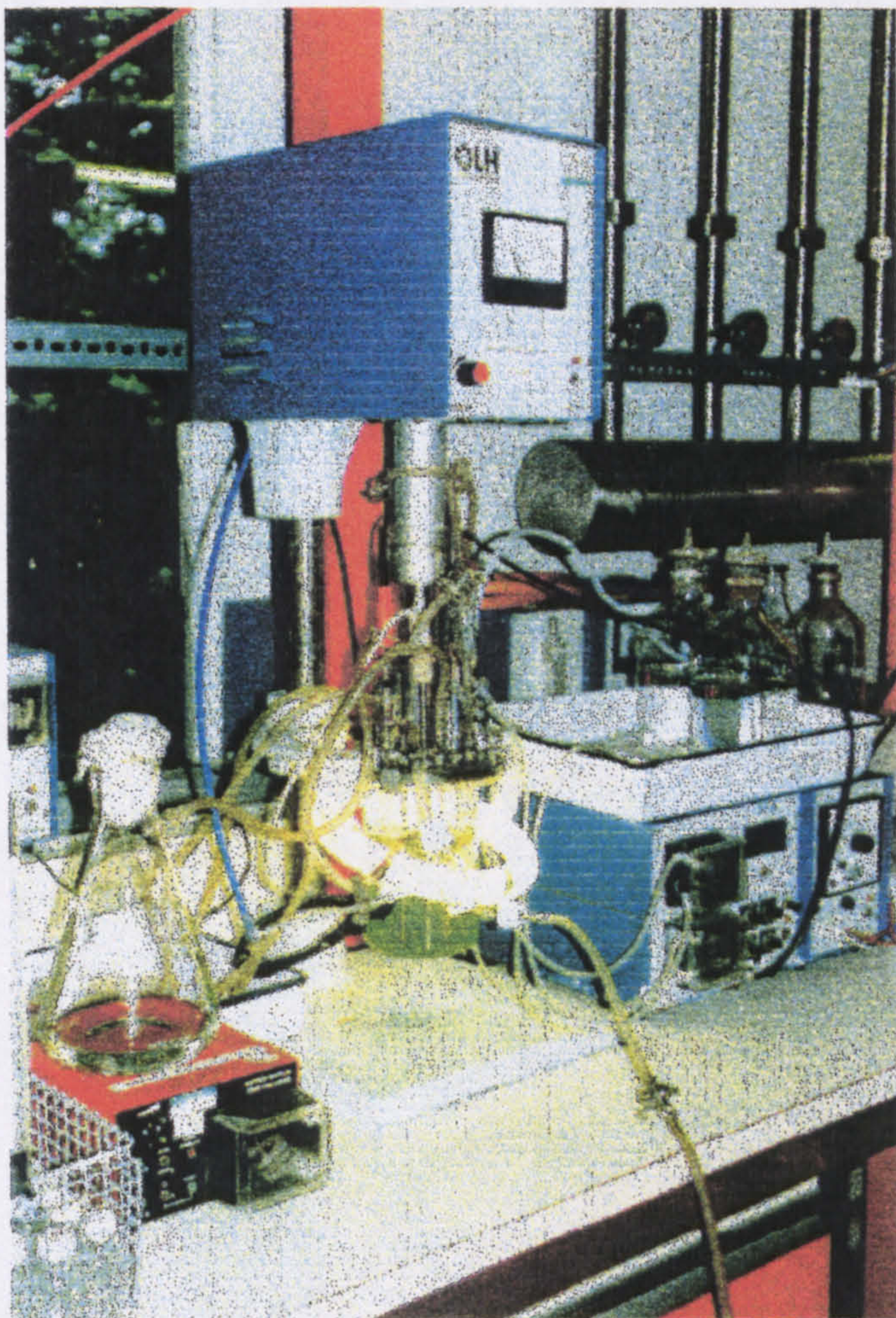


Figure 2.1: Photograph of reaction vessel showing side arm flask to the left of the picture and the acid and alkali reservoirs to the right of the picture. Below the acid and alkali reservoirs are the pH box (to the left) and the temperature box (on the right). The out flow tube to the waste reservoir is visible to the bottom of the picture. The agitator is the mechanism to which the reaction vessel is attached. The light source is clearly visible surrounding the reaction vessel.

2.2.2.2 Fermenter Culture

D. parva was grown in fermenter culture as described in section 2.2.1.1 except that the temperature was maintained at 25°C (see Figure 2.1).

2.2.3 Range of Buffers used in Batch Culture

Both algae were grown at a range of pH values in batch culture. The following buffers were used.

20 or 100 mM phthalic acid/NaOH for pH 3 and 4.5.

20 or 100 mM 2-[N-morpholino] ethane-sulphonic acid (MES)/NaOH for pH 6.

20 or 100 mM tris (hydroxymethyl) methylamine (Tris)/HCl for pH 7.5.

50 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS)/NaOH for pH 9.0 and 10.0 for *Dunaliella* and *Chlorococcum* respectively.

All measurements of pH were obtained using a HI 931400 microprocessor pH meter (Hanna Instruments).

2.4 Morphology of *C. submarinum*

Cells of *C. submarinum* pH 7.5 were grown up for a week and resuspended in fresh media of pH 5.5, 7.5 or 9.0. Daily, over the period of seven days, cells from each culture were examined under the microscope (400 times magnification). The number of normal single round cells was compared to the number of larger encysting cells. Clumping of cells was noted as were motile prolate ellipsoids. The total cell number per ml was also determined as described in section [section 3.2.10].

2.5 Chlorophyll content

2.5.1 *C. submarinum*

Chlorophyll was only readily extracted from *C. submarinum* using dimethylsulphoxide (DMSO) or DMSO mixtures (Shoaf and Lium, 1976). The method followed that of Blackwell and Gilmour (1991a). Routinely, 5 ml algal samples were centrifuged for 10 minutes at 3000 g, supernatant was immediately discarded and the pellet resuspended (by whirlmixing) in 3 ml of DMSO. The samples were left to stand for 5 minutes and then centrifuged for 10 minutes at 3000 g. The optical density (OD) of the supernatants was then measured at 645 and 663 nm and used to derive the chlorophyll content using the equation for chlorophyll concentration in 80 % acetone given by Arnon (1949). Hiscox and Israelstam (1979) confirmed that the absorption spectra of both chlorophyll *a* and *b* in DMSO were virtually identical to the spectra in 80 %

acetone. Chlorophyll content of the original sample, in μg , is then given by the equations recommended by Bruinsma (1961) for 80% acetone.

$$\{(80.2 \times \text{O D}_{663}) + (202 \times \text{O D}_{645})\} / 2 \quad (\text{Bruinsma, 1961})$$

2.5.2 *D. parva*

Chlorophyll was extracted from *D. parva* after harvesting 5 ml samples of cells at 3000 g for 10 minutes. The cells were resuspended in 1 ml of distilled water to lyse them and 4 ml of pure acetone were mixed in. Chlorophyll content of the extract was then determined by measuring OD at 645 and 663 nm (after centrifugation at 3000 g for ten minutes) using the equation given in section 2.5.1.

Lichtenthaler and Wellburn (1983) noted large variations in the estimations made for total chlorophyll by different published equations for particular solvents. The use of 80 % (v/v) acetone is prone to inaccuracy because the absorption coefficients were originally taken from Mackinney (1941) who used pure acetone. There is a considerable difference between the spectroscopic properties of 80 % (v/v) acetone and pure dry acetone (Lichtenthaler and Wellburn, 1983).

2.6 Protein Determination

Soluble protein was measured using the method described by Bradford (1976).

Bovine serum albumin (BSA) was used as the standard. The reagent used was prepared by dissolving 100 mg of Coomassie brilliant blue-G250 in 50 ml of 95 % (v/v) ethanol. 100 ml of 85 % (w/v) phosphoric acid were added. The volume was adjusted to 1 litre with distilled water.

To carry out the assay 0.1 ml of cell suspension was added to a test tube. 5 ml of the reagent were added, and the contents thoroughly mixed. After 5 minutes incubation at room temperature the absorbance was measured at 595 nm against a water blank (5 ml reagent plus 0.1 ml water). The protein content of the sample was determined from a standard curve obtained by plotting the OD₅₉₅ of standard solutions containing 0-100 µg protein ml⁻¹ (Appendix C).

2.7 Glycerol Determination

The acetylacetone method was used as described by Ben-Amotz and Avron (1978). The presence of Tris buffer interferes with this test. Therefore, the cells were either be grown in media with Hepes buffer or if grown in Tris buffered medium were rinsed several times in Hepes buffer before samples were removed.

1 ml of sample was taken and to it 100 µl of 30 % trichloroacetic acid (TCA) added. This was then whirlmixed and centrifuged at 3000 g for 10 minutes. From the clear supernatant 110 µl was removed and put into separate test tubes. 100 µl of distilled water was then added to all test tubes. A blank containing 100 µl of distilled water, 10

μl of 30 % TCA and 100 μl of Hepes medium was prepared. A set of glycerol standards were set up containing 10, 20, 30, 40 and 50 mg glycerol $100 \mu\text{l}^{-1}$. 100 μl of each standard were added to separate test tubes, then 100 μl of Hepes medium and 10 μl of TCA were also added to all standards.

1 ml of periodate reagent (130 mg sodium periodate in 180 ml 2 % acetic acid containing 15.4 g ammonium acetate, when dissolved 20 ml glacial acetic acid was added) was added to all tubes, mixed well and left for 5 min. 2.5 ml of acetylacetone reagent (2.5 ml acetylacetone made up to 250 ml with isopropanol - kept in the dark) was then added, mixed well and the test tubes were placed in a water bath at 45 °C for 15 minutes. The tubes were removed from the water bath and allowed to cool for a few minutes at room temperature. The absorbance at 410 nm was measured in a spectrophotometer for all samples and standards. The standards were used to construct a concentration curve (Appendix D).

2.8 Photosynthesis and Respiration Measurements

Oxygen evolution and uptake were routinely measured using a modified Clark oxygen electrode of the type described by Delieu and Walker (1972). The reaction chamber (working volume 2 ml) was maintained at a constant 20°C for *C. submarinum* and 25°C for *D. parva* by circulating water from a temperature controlled water bath. A Philips 12 V, 100 W projector lamp was used to illuminate the chamber (when required) with an intensity of $50 \mu\text{mols quanta m}^{-2} \text{ s}^{-1}$.

To allow the rates of oxygen evolution and uptake to be calculated, the amount of oxygen in 2 ml of air saturated medium had to be known. Increasing levels of salinity result in decreased solubility of oxygen, therefore data was required for the different salinity media used in the oxygen electrode experiments. The data of Strickland and Parsons (1968) and Gilmour (1982) were used to calculate oxygen solubilities in different salinity media and the values used are given in the equation below. Oxygen was removed from the chamber by adding sodium dithionite to allow calibration of the electrode.

Rates of oxygen evolution and uptake were measured in cells grown in pH 5.5, 7.0 and 9.0. Rates of oxygen evolution and uptake were also measured after shock experiments in which cells were resuspended in medium of a different pH from the growth medium. In all cases the chlorophyll content of cultures of either *D. parva* or *C. submarinum* was determined (section 2.5) and adjusted to 15 or 30 $\mu\text{g Chl ml}^{-1}$ by centrifugation of the required amount of culture, pouring off the supernatant and resuspending the pellet in 5 ml of fresh medium. In steady state experiments, the pH of the fresh medium was the same as the growth medium. In shock experiments, cells were resuspended in different pH media and rates of oxygen evolution and respiration were measured immediately after resuspension, and also after 3 hours. In all cases, the cells were placed in the electrode chamber and left for five minutes in the dark. The samples were then illuminated until oxygen evolution was linear and darkened again until oxygen uptake was linear. The rate of oxygen evolution or uptake was calculated using the following equation:

$$\text{Respiration rate} = \frac{\text{standard range}}{\text{range}} \times \frac{\text{number of units}}{\text{time}} \times \frac{60}{\mu\text{g protein present sample}}$$

standard: oxygen solubilities in different salinity media.

D. parva (1.5 M NaCl) = 0.180 $\mu\text{moles O}_2 \text{ ml}^{-1}$ at 25°C

Strickland and Parsons (1968).

C. submarinum (0.25 M NaCl) = 0.255 $\mu\text{moles O}_2 \text{ ml}^{-1}$ at 20°C

Gilmour (1982).

range: units taken from calibration.

number of units: number of units covered in a certain period of time either with or without light.

time: the time length in minutes for which the sample was measured.

60: this converts the time from minutes to hours.

$\mu\text{g protein present in sample}$: this relates to the chlorophyll content of the sample for example 30 $\mu\text{g Chl ml}^{-1}$ = 0.060 (doubled as there is a 2 ml sample).

The rate of respiration was taken as being the rate of oxygen uptake in the dark. To calculate the photosynthetic rate, it was assumed that respiration in the light was equal to dark respiration. The rate of photosynthesis was therefore equal to oxygen evolution in the light plus oxygen uptake in the dark. This assumption is probably not completely valid, but since respiratory rates are generally only a small proportion of

photosynthetic rates the error involved is likely to be small (Jackson and Volk, 1970; Edwards and Walker, 1983).

2.9 Estimation of Cell Number

Cell number was determined by counting cells in a haemocytometer chamber (Weber Scientific International Ltd, England). The chamber had a depth of 0.1 mm and on the surface of the chamber a grid of twenty-five squares totalling 1 mm² was imprinted. Of these twenty-five squares five diagonal squares were counted. Each of these squares was divided into sixteen smaller squares (1/400 mm²). Capillarity carried the sample across to fill the grid and the volume added was sufficient to partially fill the moat surrounding the chamber. Cells were allowed to settle before counting commenced. The method employed followed that of Penn (1991). At least four grids or a total of at least 200 cells were counted to ensure accuracy (Harris, 1989). Cells were not so dense that they were difficult to distinguish individually, or so sparse that they were difficult to find (Penn, 1991).

As the *D. parva* cells were motile they were killed before counting could be undertaken. A 300 µl sample of the alga was put into a fresh test-tube and 30 µl of Grams' iodine solution (2 g of potassium iodide dissolved in 300 ml of distilled water and then 1 g of iodine added) was added and whirlmixed.

For both algae species, cells were concentrated to a range of chlorophyll contents: 5, 10, 15, 20, 30, 45 and 60 $\mu\text{g Chl ml}^{-1}$. Five replicates of each sample were measured, and for each of the pH levels six samples were studied. The count for each individual replicate was multiplied by 5×10^4 to obtain a final concentration in cells per ml. This calculation took into account the fact that only five of the twenty-five squares were counted, hence the multiplication by 5. The results were then multiplied by 10^4 to take into account the total volume of the $0.1 \times 1 \text{ mm}^2$ grid.

$$\text{i.e. } 1 \text{ mm} \times 1 \text{ mm} \times 0.1 \text{ mm} = 0.1 \text{ mm}^3$$

$$0.1 \text{ mm}^3 \times 10^{-3} = 1 \times 10^{-4} \text{ cm}^3 \text{ (ml)}$$

For *D. parva* the dilution with iodine had to be taken into account so these results were then multiplied by 1.1.

2.10 Estimation of Cell Volume by Micrometric Measurements

The microscope was calibrated using an eyepiece scale and a microscope slide micrometer (100 units = 1 mm).

D. parva: Cell volume was determined by measuring the short (a) and long (b) axes of 50 cells; since the cells are prolate ellipsoids the volume equals $\frac{4}{3}\pi a^2b$ (Ginzburg and Ginzburg, 1993, Zmiri *et al.*, 1984).

C. submarinum: Cell volume was determined by measuring the radius (r) of 50 cells and as the cells are spherical the volume equals $\frac{4}{3}\pi r^3$.

2.11 Silicone Oil Technique

Centrifugation through silicone oil was used to completely separate cells from medium (Gimmler and Schirling, 1978). A range of Dow Corning silicone oils were obtained from Merck (Lutterworth) and different oil densities were produced by mixing individual silicone oils. In the experiments different oils were used depending on the algae studied and pH of the sample. Cell samples of the desired chlorophyll content (with no isotopes added) were spun through the oil mixture to establish the correct density of the oil, thus allowing the cells to pass through the oil during centrifugation and produce a pellet, but restrict mixing with the medium (Figure 2.2).

D. parva required pure 710 oil while *C. submarinum* required a mix of 1:5 of 200/ICS and 550 oils respectively. The required concentrations of chlorophyll in each sample can be seen in Table 2.1.

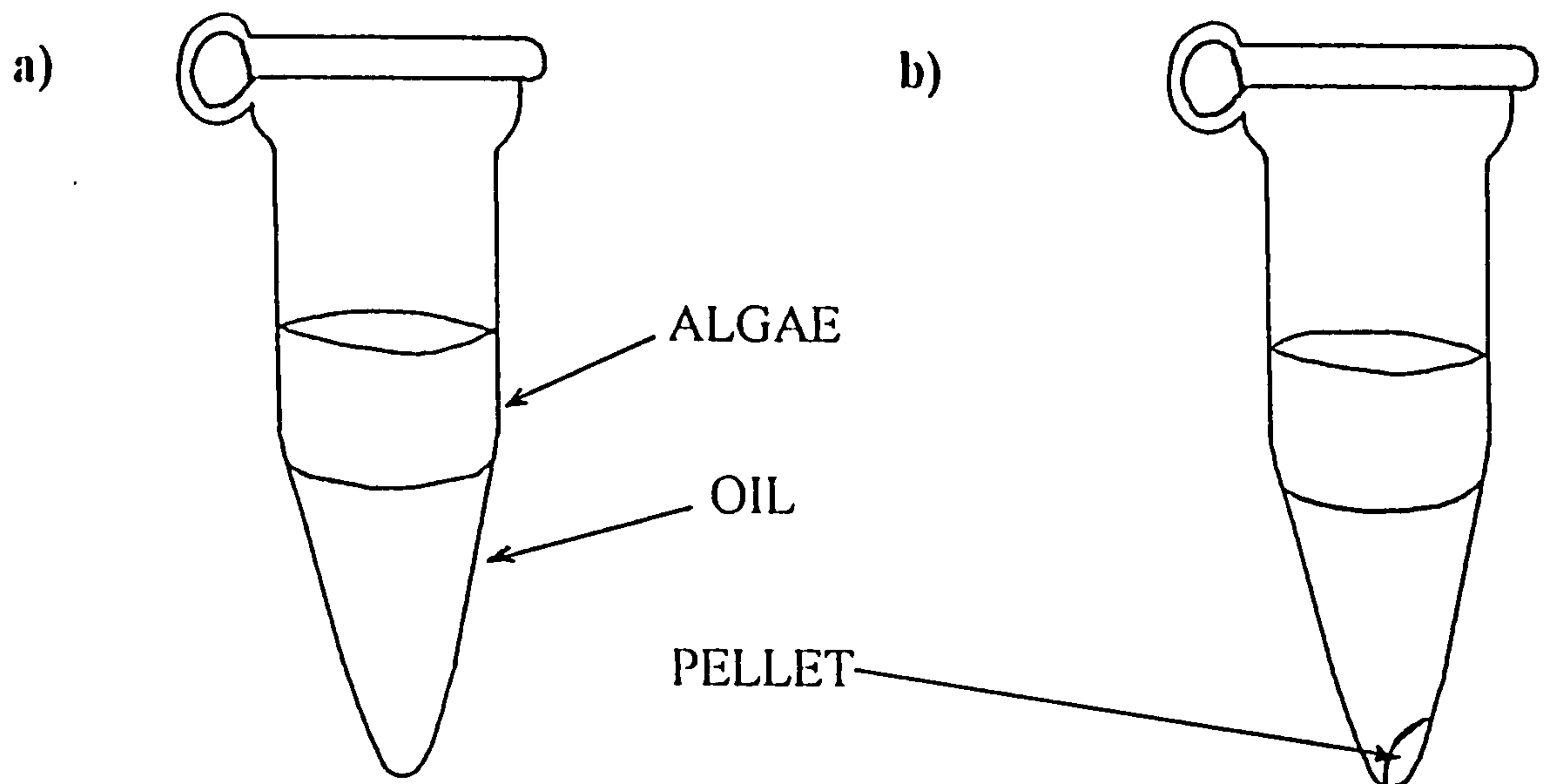


Figure 2.2: a) The cell sample was layered on top of the silicone. b) After centrifugation a pellet was formed and the medium remained on top of the oil.

2.11.1 Determination of Cell Volume

Two samples of concentrated cells, each of one ml, were placed in 1.5 ml Eppendorf tubes. The concentration of the cells depended on the species of the algal sample and pH of the growth medium of the sample (Table 2.1). To one sample 11 μl of $^3\text{H}_2\text{O}$ (1850 kBq ml^{-1}) was added to give 20 kBq ml^{-1} and to the second sample 20 μl of ^3H -dextran (MW = 70000, 740 kBq ml^{-1}) was added to give 14.5 kBq ml^{-1} . Both samples were carefully whirlmixed. After 10 minutes incubation at room temperature, triplicate samples (0.3 ml) were taken from each Eppendorf and layered onto 0.3 ml of

pH	$\mu\text{g Chl ml}^{-1}$	
	<i>D. parva</i>	<i>C. submarinum</i>
5.5	50	60
6.0	50	60
7.5	50	60
9.0	60	50
10.0	60	50

Table 2.1: Table of chlorophyll concentrations used in the Silicone Oil experiments for *D. parva* and *C. submarinum* cells grown in a range of pH media.

silicone oil in a fresh Eppendorf tube. The samples were then centrifuged in a microcentrifuge (MSE, MicroCentaur) at 13,000 *g* for 2 minutes. From the aqueous supernatant fraction, samples (50 μl) were taken and added to 5 ml scintillation fluid (Safe Fluor S, Lumac LSC B.V., The Netherlands) in a scintillation vial and counted in a Beckman LS 1801 Liquid Scintillation Counter. The lower portion of these Eppendorf tubes, which contained the pellets of alga, were cut off within the oil layer and placed cut end down in Eppendorf tubes containing 0.3 ml distilled water. They were then centrifuged for 20 seconds to remove the pellets from the tips and the tips were discarded. The pellets were resuspended in the water, the whole sample was pipetted out and added to scintillation vials containing 5 ml scintillation fluid. The vials were counted in the liquid scintillation counter.

The $^3\text{H}_2\text{O}$ was evenly distributed throughout the pellet, whereas the ^3H -dextran was only found in the spaces between the cells and the pellet (Ginzburg, 1969). The pellet volume and the extracellular volume (ECV) were calculated from the ratio of $^3\text{H}_2\text{O}$ and ^3H -dextran, respectively, in the cell and supernatant fraction using the following equations (Hard and Gilmour, 1996):-

$$\text{Pellet Volume } (\mu\text{l}) = \frac{{}^3\text{H}_2\text{O dpm in pellet}}{{}^3\text{H}_2\text{O dpm in supernatant} \times 6} \times 300$$

$$\text{Extracellular Volume } (\mu\text{l}) = \frac{{}^3\text{H - Dextran dpm in pellet}}{{}^3\text{H - Dextran dpm in supernatant} \times 6} \times 300$$

The intracellular volume (ICV) was calculated by subtracting the ECV from the total pellet volume (Rottenberg, 1979). If the cell number was also determined on a parallel sample, the average volume of an individual cell could be calculated. It was established ^3H -dextran was not a reliable probe for the measurement of extracellular volume (section 4.2.1) and thus ^3H -dextran measurements were not taken for all samples. A ratio was established between extracellular volume and intracellular volume for both algae grown at pH 7.5. This was then used in all of the silicone oil experiments. Section 4.2.1 presents data which shows that the extracellular volume has very little effect on the membrane potential and internal pH calculations although the errors in the extracellular measurements may cast doubt over the cell volume calculations (section 3.2.6.2).

2.11.2 Measurement of Membrane Potential

The membrane potential was determined using $^3\text{H-TPP}^+$ (tetraphenylphosphonium) as described by Rottenberg (1979; 1989). The experimental procedure was similar to the method used to determine ICV (section 2.11.1) and is described below.

1. 5 μl of 800 kBq $^3\text{H-TPP}^+$ was added to 1 ml of concentrated cells to give a final concentration of 20 kBq ml^{-1} $^3\text{H-TPP}^+$.
2. The cell suspension was mixed and incubated at room temperature for either 3 hours or 4 hours for *D. parva* or *C. submarinum* respectively..
3. The cells were centrifuged through silicone oil as in section 2.11 and the same amounts of supernatant and pellet were taken for counting.

The membrane potential was calculated as follows:-

- a) Dpm $^3\text{H-TPP}^+$ of supernatant was divided by 50 = dpm $^3\text{H-TPP}^+$ in 1 μl = A.
- b) Multiply A by extracellular volume in μl (calculated from parallel samples treated with $^3\text{H}_2\text{O}$ and $^3\text{H-dextran}$) = $^3\text{H-TPP}^+$ within the pellet which is outside the cells = B.

c) $\text{Dpm } ^3\text{H-TPP}^+$ in the pellet minus **B** and divided by intracellular volume in μl (calculated from parallel samples treated with $^3\text{H}_2\text{O}$ and $^3\text{H-dextran}$) = $\text{dpm } \mu\text{l}^{-1}$ cell volume = **C**.

d) Ratio of **C/A** = concentration of TPP^+ inside the cell (a_i) / concentration of TPP^+ outside the cells (a_o).

Using the Nernst equation:-

$$\Delta\psi \text{ (mV)} = (-RT/ZF) \ln (a_i / a_o)$$

where,

$$R = 8.3143 \text{ Joules mol}^{-1} \text{ K}^{-1}$$

$$T = 303 \text{ K (30 } ^\circ\text{C)}$$

$$F = 96.487 \text{ Joules ml}^{-1} \text{ mV}^{-1}$$

$$Z = 1 \text{ (charge on ionic species).}$$

At 30 $^\circ\text{C}$ and converting from \ln to \log ($\times 2.303$):-

$$\Delta\psi \text{ (mV)} = -58.8 \times \log a_i / a_o$$

2.11.3 Determination of Internal pH

The internal pH was measured using a weak acid or base (Rottenberg, 1979; 1989; Kashket, 1985). In order to obtain a measurable accumulation, a weak acid (^{14}C -benzoic acid) was used when the external pH was lower than pH 7.0 and a weak base (^{14}C -methylamine) was used when the external pH was higher than pH 7.0.

The silicone oil method used was identical to that used for ICV calculation (section 2.11.1) and membrane potential determination (section 2.11.2), except that 5 μl of 40 kBq $10\mu\text{l}^{-1}$ ^{14}C -methylamine was added to give a final concentration of 20 kBq ml^{-1} and left for 40 minutes in *D. parva* and 30 minutes in *C. submarinum*. The calculations were exactly the same as those used in section 2.11.2 to calculate the ratio a_i/a_o . To determine the internal pH from the a_i/a_o ratio calculated from ^{14}C -methylamine, two equations can be used (below).

If the pK of the probe is more than 1.5 units above the external pH then:-

$$\Delta\text{pH} = -\log(a_i / a_o)$$

If the pK of the probe is less than 1.5 units above pH outside the cells then

$$\text{pH}_i = \log [a_i / a_o (10^{-\text{pK}} \times 10^{-\text{pH}_o}) - 10^{-\text{pK}}]$$

$$\text{pK of methylamine} = 10.6$$

5 μl of 40 kBq $10 \mu\text{l}^{-1}$ ^{14}C -benzoic acid to give a final concentration of 10 kBq μl^{-1} was used for low external pH values and left to incubate for 30 minutes in *D. parva* and 360 minutes in *C. submarinum*. There are also two equations involved in the calculations (below).

If the pK of the weak acid is more than 1.5 units below the external pH, then the following equation is utilised.

$$\Delta\text{pH} = \log (a_i/a_o)$$

If the pK of the weak acid is less than 1.5 units below the external pH, this alternative equation is used.

$$\text{pH}_i = \log [a_i/a_o(10^{\text{pK}} - 10^{\text{pH}_o}) - 10^{-\text{pK}}]$$

pK of benzoic acid is 4.2

2.12 Intact Chloroplast Isolation

This procedure was based on the method described by Mason *et al.* (1991).

Cells were grown for eight days in batch culture or in the fermenter and harvested by centrifugation (Beckman, J2-21 Centrifuge, rotor JA-14) at 6000 g for 10 minutes. The pellets were washed in 100 ml of 20 mM Hepes-NaOH (pH depending on the original medium pH) and were resuspended in 2 ml ice-cold Hepes-NaOH. Subsequent steps were carried out at 4 °C. Cells were diluted with 10 ml breaking buffer (300 mM sorbitol-*C. submarinum* or 600 mM sorbitol-*D. parva*, 50 mM Hepes-NaOH, 2 mM Na-EDTA, 1 mM MgCl₂, 1 % BSA) and broken immediately. Cells of both algae were lysed using an ultrasonic disintegrator (MSE Soniprep 150). The sonication vessel was surrounded by an ice-water mixture. *D. parva* cells were disrupted by sonication for 10 seconds at a power setting of 7 microns, while *C. submarinum* cells were disrupted for 15 minutes at a power setting of 22 microns, interspersed with regular periods of cooling to prevent local warming of the suspension. The lysate was then centrifuged in a JA-20 rotor for 15 minutes at 4000 g to pellet whole cell and intact chloroplasts. No breaking was applied at the end of the spin to prevent shattering any of the intact chloroplasts. This pellet was resuspended in 2 ml breaking buffer and layered onto discontinuous Percoll gradients (20, 45, 60 % Percoll). A 15 minute centrifugation of the gradients was carried out in a JA-20 Rotor at 6000 g, again no breaking was applied so as not to disrupt the gradient. The 20 to 45 % interface areas were collected and diluted with 20 ml breaking buffer. This was then centrifuged at 6000 g for 10 minutes in a JA-20 rotor and the pellets resuspended in 1 ml resuspension buffer (300 mM sorbitol - *C. submarinum* or 600 mM - *D. parva*, 50 mM Hepes, 2 mM EDTA, 1 mM MgCl₂, 1 mM KH₂PO₄).

2.12.1 Verification of Intact Chloroplasts

2.12.1.1 Microscope

The presence of intact chloroplasts was confirmed by examination under a microscope at 1000 times magnification with the aid of cedar oil (Sigma) and the phase contrast setting.

2.12.1.2 NaH¹⁴CO₃ Fixation

This method was based on one presented by Goyal *et al.* (1988). A sample of 1 ml intact chloroplasts in resuspension buffer was added to a 1.5 ml Eppendorf tube. A sample of 1 ml of intact cells which had been resuspended in 1 ml resuspension buffer at the step prior to sonication were added to a second Eppendorf. To each of these Eppendorfs 10 µl of 37 kBq NaH¹⁴CO₃ were added (in fume cupboard). The samples were stood in good illumination for 1 hour and then 0.1 ml of 30 % trichloroacetic acid (TCA) was added to both Eppendorfs. Both samples were left for 1-2 minutes with their tops off in a fume cupboard. After this time the tops were again replaced and whirlimixed. The tops were removed and the samples left overnight in the fume cupboard. After twelve hours incubation the tops were replaced and samples whirlimixed. The whole sample was removed and added to 5 ml scintillation fluid and counted in the scintillation counter.

2.12.2 Internal pH and $\Delta\psi$ Measurements of Intact Chloroplasts

The methods used were as described previously (sections 2.11.2 and 2.11.3). The isotopes were added to the intact chloroplasts and left to stand for 30 minutes before being centrifuged through silicone oil. Dow Corning 550 oil was used for *D. parva* and a mixture of Dow Corning 550 and 200 ICS (5:1) oils for *C. submarinum*.

2.13 Enzyme activities of *Dunaliella parva* and *Chlorococcum submarinum* grown at different pH values

2.13.1 Preparation of cell free extract

Algae were grown in fermenter or batch culture (section 2.2) and the chlorophyll content of the cells determined (section 2.5). The cells were harvested by centrifugation at 3000 g in a bench centrifuge for 10 minutes and resuspended in 10 ml of 5 mM HEPES-NaOH buffer pH 7.5 containing 1 mM dithiothreitol (DTT). They were then placed immediately on ice. *D. parva* cells were disrupted using an ultrasonic disintegrator (MSE Soniprep 150). The sonication vessel was surrounded by an ice-water mixture and the cells disrupted by sonication for 10 seconds, divided into two 5 second bursts to prevent local warming of the suspension. The probe (1 cm diameter) was used at a power setting of 7 microns. The cells were examined under a microscope at 400 times magnification to check for cell breakage and kept on ice.

C. submarinum cells were disrupted using a French Pressure Cell Press (Simoamico, SLM Instruments, INC.). A 40,000 PSI pressure cell was used and the sample was subjected to a high ratio selector and a pressure of 1,500 PSI. These cells were also examined under a microscope at 400 times magnification to check for total breakage. The cells were kept on ice. Cell debris was removed by centrifugation at 4000 g for 15 minutes at 4 °C in a Beckman J2-21 centrifuge. The supernatant fluid obtained was referred to as the cell-free extract. It was kept on ice and used for measurements within five hours.

2.13.2 General assay conditions

Continuous assays of enzyme activity were carried out using a Ultraspec II LKB Biochrom spectrophotometer, the temperature of the cuvettes was maintained at room temperature (~ 20-25 °C). Quartz cuvettes (1.0 ml or 4.0 ml) with 1 cm light path were used in all cases. For all assays the reaction rate was initially linear and proportional to the amount of extract present. Any activity found before the addition of the substrate was subtracted from the reaction rate. *D. parva* samples were concentrated to 15 µg Chl ml⁻¹ and *C. submarinum* samples to 30 µg Chl ml⁻¹.

Activity measurements were taken from both pH shocked cells and pH adapted cells (section 5.2). The pH adapted cells were centrifuged and resuspended in media of the same pH. The activity of these cells was measured immediately. The pH-shocked cells were resuspended in either pH 5.5, 7.5 or 9.0 media. These cells were centrifuged and resuspended in their new medium for three hours before the activity rates were measured.

The results were calculated using the equation:

$$b = \frac{\Delta A \times 1000}{\epsilon \times d \times \Delta t \times \phi} \quad \mu\text{mol} \times \text{min}^{-1} \times \text{l}^{-1} \text{ (U/l)} \quad (\text{Bergmeyer and Grabl, 1983}).$$

Where:

ΔA : Absorbance

ϵ : Adsorption coefficient

d : Light path, mm

Δt : time, minutes

$\phi = \frac{v}{V}$ Volume of sample used in assay
Total assay volume

2.13.3 Fumarase



The method of Hill and Bradshaw (1969) was used to detect the formation of fumarate from malate.

The reaction mixture contained (values in ml):-

	<i>D. parva</i>	<i>C. submarinum</i>
50 mM Tris/HCl	1.5	1.5
50 mM L. malate (sodium salt) (pH 7.5)	0.2	0.2
Cell free extract	0.4	0.2
Water to final volume	3.0	3.0

The reaction was initiated by the addition of 0.2 ml cell free extract, and the increase in absorption at 240 nm (with deuterium lamp) and was recorded against a water blank. Enzyme activity is expressed as nmole fumarate produced $\text{min}^{-1} \text{mg. protein}^{-1}$.

The absorption coefficient for fumarate at 240 nm, pH 7.5 is $0.244 \text{ l x mmole}^{-1} \text{ mm}^{-1}$. Differences in pH appeared not have any effect on this absorption coefficient (Stitt, 1984).

2.13.4 Malate Dehydrogenase



The assay was based on the method described by Reeves *et al.* (1971). The assay mixture contained (values in ml):-

	<i>D. parva</i>	<i>C. submarinum</i>
50 mM Tris/HCl	1.5	1.5
1.5 mM NADH	0.05	0.05
7.5 mM OAA: pH 7.5	0.2	0.2
Cell free extract	0.05	0.01
water to final volume	3.0	3.0

The oxaloacetate was prepared fresh daily because it is unstable.

The reaction was started by the addition of oxaloacetate and the decrease in absorbance at 340 nm was followed against a water blank. Enzyme activity is expressed as $\mu\text{mole NADH oxidised min}^{-1} \text{ mg protein}^{-1}$.

The extinction coefficient of NADH at 340 nm is $6.22 \times 10^3 \text{ l mole}^{-1} \text{ cm}^{-1}$.

2.13.5 Dihydroxyacetone Reductase (DHA Reductase)



The assay for this enzyme followed the method described by Gimmler *et al.* (1984).

The reaction mixture contained (values in ml):-

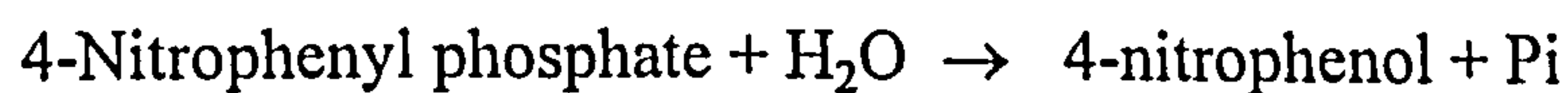
	<i>D. parva</i>	<i>C. submarinum</i>
31 mM Tris/HCl	1.5	-
0.1 mM NADPH	0.1	-
4.5 mM DHA	0.4	-
Cell free extract	0.4	-
water to final volume	3.0	-

The reaction was started by the addition of DHA and the decrease in absorbance at 340 nm was followed against a water blank. Enzyme activity is expressed as $\mu\text{mole NADPH oxidised min}^{-1} \text{ mg protein}^{-1}$.

The extinction coefficient of NADPH at 340 nm is $6.22 \times 10^3 \text{ l mole}^{-1} \text{ cm}^{-1}$.

2.13.6 Acid Phosphatase

This assay also follows the method described by Gimmler *et al.* (1984).



(pH 4.9 acid phosphatase enzyme) (Moss, 1984)

The reaction mixture contained (values in ml):-

	<i>D. parva</i>	<i>C. submarinum</i>
90 mM Citrate/NaOH	1.5	1.5
20 mM P-nitrophenylphosphate	0.2	0.2
Cell free extract	0.5	0.1
water to final volume	3.0	3.0

The reaction mixture was incubated at 30 °C for 25 minutes. The spectrophotometer was blanked at 405 nm with water and an increase in absorption was measured against the control to which no p-nitrophenylphosphate had been added before incubation. A calibration curve was produced to enable the calculation of enzyme activity which was expressed as $\mu\text{mole p-nitrophenol produced min}^{-1} \text{ mg protein}^{-1}$.

2.14 Statistics

Throughout at least three independent experiments were averaged to give results shown. Standard error of the means are given and error bars represent standard error of the means. If no errors are shown, then the data is from one representative experiment. In this case the experiment will have been repeated on at least two occasions with the same trend evident.

Some of the results produced were subjected to analysis of variance tests to establish their significance. Probability (P) was considered to be significant at 95% ($P < 0.05$). Data was analysed using Microsoft Excel v5.

pH tolerance of *Dunaliella parva*
and *Chlorococcum submarinum*.

CHAPTER THREE

3.1 Introduction

Measurement of cell growth rate is a relatively simple, but very effective method of determining the tolerance of an organism to an environmental stress. *Dunaliella parva* has been found by Gimmler *et al.* (1988) to grow equally well across the pH range of 5.5 to 8.0. The effect of pH on the growth rate of *Chlorococcum submarinum* has been studied by Blackwell and Gilmour (1991a) who found this alga to be viable in both high and low external pH. This area of study is fundamental to the whole project because if the cells are unable to grow in the extreme pH media then no measurements at all can be taken.

The measurement of the absorbancies for chlorophylls *a* and *b* in algae after extraction with acetone or DMSO is a simple and rapid method to determine growth. Unfortunately this method is regarded as inaccurate by many scientists. There is some debate about the accuracy of the absorption coefficients produced by MacKinney in 1941 although these equations recommended for the estimation of chlorophylls in 80% acetone are still in use today (Brunisma, 1961). The total chlorophyll content measured in a sample can vary depending on the spectrophotometer used. If the same spectrophotometer is used throughout, then although the readings may not be precise, the results from the samples are comparable. This chlorophyll content measurement method is also not an accurate measurement of cell number. Cell number can vary depending on the environmental conditions for growth and in many, although not all

cases, the larger the cell, the greater the chlorophyll content and therefore an over estimation of cell number would be made.

Measurement of cell number is a second method for the estimation of growth rate. This method is a more accurate method than chlorophyll measurements but when palmelloid cells are found (four cells inside a single mother wall), cell counts are less accurate (Harris, 1989). If the cells become stressed then clumping or encysting may occur making counts impossible. This method is also very time consuming and is prone to operator error.

Biomass measurements are another alternative. These are usually expressed as dry weights and therefore not suitable for many experiments that require instant measurements. The sample of interest requires a drying time, during which the culture from which the sample was removed will continue to change. This method would also not be suitable for samples grown with salt in the media unless the cells were washed in salt free medium which is not feasible for many cells grown in high salinity.

Photosynthesis and respiration measurements are good methods for showing the detrimental effects of external factors which affect algal growth. The fact that the pH of the growth medium has an effect on the availability of CO₂ for photosynthesis and respiration could be a complicating factor (Borowitzka and Borowitzka, 1988). The rapid adjustment of respiration and photosynthesis is crucial to survival and growth in environments prone to fluctuations (Blackwell and Gilmour, 1991c).

3.2 Results and discussion

3.2.1 Maintenance of Medium pH using Buffers

D. parva

For *D. parva* grown in 20 mM Tris buffered growth medium (Appendix A), the original pH was not maintained: After only eight hours of growth the pH of the 20 mM Tris medium had increased significantly from pH 7.5. This was not acceptable for the experiments to be undertaken.

An experiment was designed with *D. parva* cultures which were set at pH 7.5 with 20, 50 or 100 mM Tris buffer to establish the most reliable concentration of the buffer. The pH of each culture was measured regularly. Table 3.1 shows clearly that 100 mM Tris buffer held the growth media at the desired pH over the time period studied. The 20 and 50 mM Tris buffered media allowed the media pH to rise and therefore these two lower concentration buffers were of no use.

After establishing that the highest tested level of Tris buffer was required to maintain the desired pH of 7.5 for *D. parva*, a second experiment was devised to test the effect of varying concentrations of Tris buffer on the growth of the alga.

Days after inoculation	20 mM Tris Buffer (pH units)	50 mM Tris Buffer (pH units)	100 mM Tris Buffer (pH units)
0	7.5	7.5	7.5
12	8.21 ± 0.10	7.69 ± 0.01	7.58 ± 0.02
14	8.08 ± 0.10	7.63 ± 0.01	7.51 ± 0.01
18	8.16 ± 0.10	7.68 ± 0.01	7.51 ± 0.01
21	8.10 ± 0.13	7.71 ± 0.01	7.53 ± 0.01

Table 3.1: Effect of the concentration of Tris buffer on the pH of medium in which *Dunaliella parva* was grown. The pH value was initially set at pH 7.5 in all cases. The mean ± standard error from three replicates is shown for each experiment.

D. parva cells were inoculated in standard *Dunaliella* medium (Appendix A). Flasks contained 20, 50 or 100 mM Tris buffer and the growth of the cells was monitored over a 21 day period using chlorophyll determination (Section 2.5.2).

Table 3.2 shows that growth occurred in all flasks. Highest levels of growth were found in the flasks with the addition of 100 mM buffer and lowest levels of growth in 20 mM Tris medium. Therefore, the high levels of Tris buffer did not have a toxic effect on the alga, and in fact growth was greatest in high levels of Tris. The high growth rates with

Time (Days)	$\mu\text{g Chl ml}^{-1}$		
	20 mM Tris	50 mM Tris	100 mM Tris
12	14.07 ± 1.19	15.71 ± 0.80	15.80 ± 2.73
14	13.32 ± 0.78	14.07 ± 1.19	17.71 ± 0.77
18	18.56 ± 0.48	21.04 ± 0.50	25.54 ± 1.89
21	19.99 ± 0.54	24.94 ± 0.90	31.62 ± 0.04

Table 3.2: The effect of different concentrations of Tris buffer on growth of *Dunaliella parva*. The mean \pm standard error from three replicates is shown for each experiment.

100 mM Tris buffer were likely to be a result of the medium maintaining a near optimum growth pH for the algae.

C. submarinum

C. submarinum was found to maintain the required pH with 20 mM Tris buffer. This may be because *C. submarinum* has a thick cell wall and is unable, as easily as *D.*

parva, to excrete substances that change the external pH. Another explanation may be that *C. submarinum* is better able to adapt to changes in external pH and does not require to try and alter the external pH to be more suitable.

3.2.2 Use of Fermenter to Maintain pH

The pH of samples of media were measured after they had been autoclaved. The media for both *D. parva* and *C. submarinum* set at pH 9 and 10 using CAPS buffer was found to precipitate during autoclaving, and even when the buffer was added to the rest of the media after autoclaving, precipitation still occurred. Filter sterilisation was also unsuccessfully tried as precipitation also occurred when the different chemical compounds were mixed together. Drifting occurred in pH values of all media during autoclaving. This was more pronounced in the high pH media.

Consequently algae were grown in a fermenter set up as a batch culture with the automatic addition of acid or alkali to overcome the problem of media precipitation at high pH levels. Using this apparatus the pH of the media could also be accurately monitored. The medium, with Tris buffer, was added to the fermenter and after about ten days of algal growth, once the algae had reached the stationary phase, the pH of the fermenter culture was increased or decreased. Fortunately no precipitation of the medium occurred. Also with this method the same buffer (Tris) was employed for all pH values, thus reducing differences in growth resulting from the use of a variety of buffers.

3.2.3 Growth of *D. parva* and *C. submarinum* over a Range of pH Values

Initial measurements for both algae were made in normal batch cultures (i.e. not in the fermenter) at pH 5.5, 7.5 and 9.0 (sections 2.2.1.1 and 2.2.2.1) over a period of 21 days. Chlorophyll measurements were taken regularly as a measure of growth (section 2.5). Although precipitation had occurred in the flasks with media at alkaline pH it was decided to continue to try and obtain growth curves in batch culture. A growth curve collected from algae grown in the fermenter would entail more problems. In the fermenter the algae were grown at pH 7.5 until the cell density was high enough to tolerate a change in the pH of the medium. The media pH change took place over several days until the desired pH was reached. Therefore the initial chlorophyll readings for a growth curve would be high with growth at pH 7.5, drop down as the pH of the medium was changed and then increase again as the cell number in the fermenter increased again. This would not produce the traditional type of growth curve.

It must be taken into account that any reduction in growth for cells resuspended in extreme pH media may not have been a direct result of the extreme pH at which the cells were stressed, but may have been due to the lack of a particular nutrient(s) which had precipitated out of the medium.

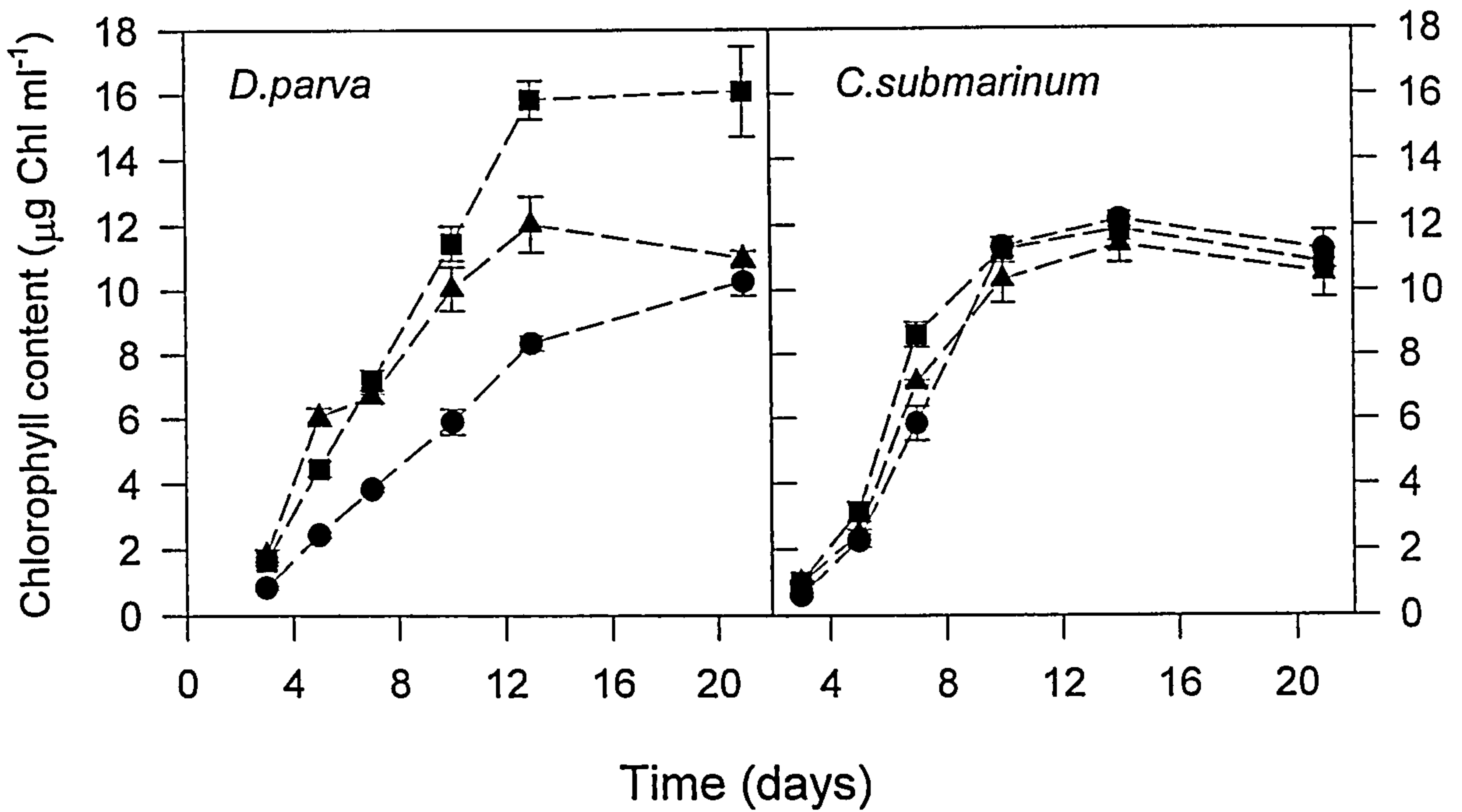


Figure 3.1: Growth curves of *D. parva* and *C. submarinum* at pH 5.0 (●), 7.5 (■), 9.0 (▲).

D. parva (Figure 3.1)

D. parva was found to grow least well in pH 5.5 media. Low growth rates were measured at this pH over the 21 days of study, however, growth levels did increase steadily over the whole three week period.

Highest levels of growth were found in cells with an external pH of 7.5. Chlorophyll contents increased over time, reaching a maximum after two weeks. There was no decrease in chlorophyll after three weeks of growth. At day 21 the error margin was seen to be large.

The levels of growth found for *D. parva* in pH 9.0 media followed the same pattern as cells grown in pH 5.5 and 7.5, up to the 14 day measurement. pH 9.0 grown cells produced higher chlorophyll levels than pH 5.5 grown cells, but lower levels when compared to pH 7.5 grown cells. After 14 days the chlorophyll measurements of pH 9.0 *D. parva* cells showed a decrease ($P = 0.503$) to a level very close to the 21 day measurement for pH 5.5 grown cells.

Based on the three external pH values selected the optimum pH for growth of *D. parva* is pH 7.5. This may be the correct conclusion, but the reduced growth rates found in the pH 9.0 stressed cells may be a result of the precipitation of the medium and the cells lacking the(se) nutrient(s) for growth. It is possible to conclude, though, that the ideal time for cell harvest is after 14 days for all three pH values tested.

Gimmler *et al.* (1988) found similar growth rates for *D. parva*. They established that *D. parva* grew equally well in the pH range between 5.5 and 8.0 although they also found that growth and photosynthesis were significantly reduced at more extreme pH values.

C. submarinum (Figure 3.1)

C. submarinum grew equally well in pH 5.5, 7.5 and 9.0 media over the 21 day period and followed the same pattern of growth. After ten days the chlorophyll measurements for all three pH values tested were not significantly different, the greatest difference being between the pH 5.5 cells and the pH 7.5 cells ($P = 0.215$). Figure 3.1 also shows clearly that the cells peaked after fourteen days growth. After this time the chlorophyll levels decreased.

The conclusion that can be drawn from this experiment is that pH values in the range from 5.5 to 9.0 have no effect on growth of *C. submarinum*. These results concurred with those of Blackwell and Gilmour (1991a) who found growth of *C. submarinum* to be unaffected by pH across the range 4.5 to 10.0.

3.2.4 Electron micrographs of *D. parva* and *C. submarinum* grown at pH 5.5, 7.5 and 9.0.

Electron micrographs were taken in the Electron Microscope Unit at Sheffield University of both algae grown at pH 5.5, 7.5 and 9.0. The methods used to prepare both algae for the electron microscope are given in Appendix E. It is not possible to draw conclusions about cell size from these electron micrographs due to cell shrinkage occurring during fixation. Also each electron micrograph is of only one cell which may not be representative.

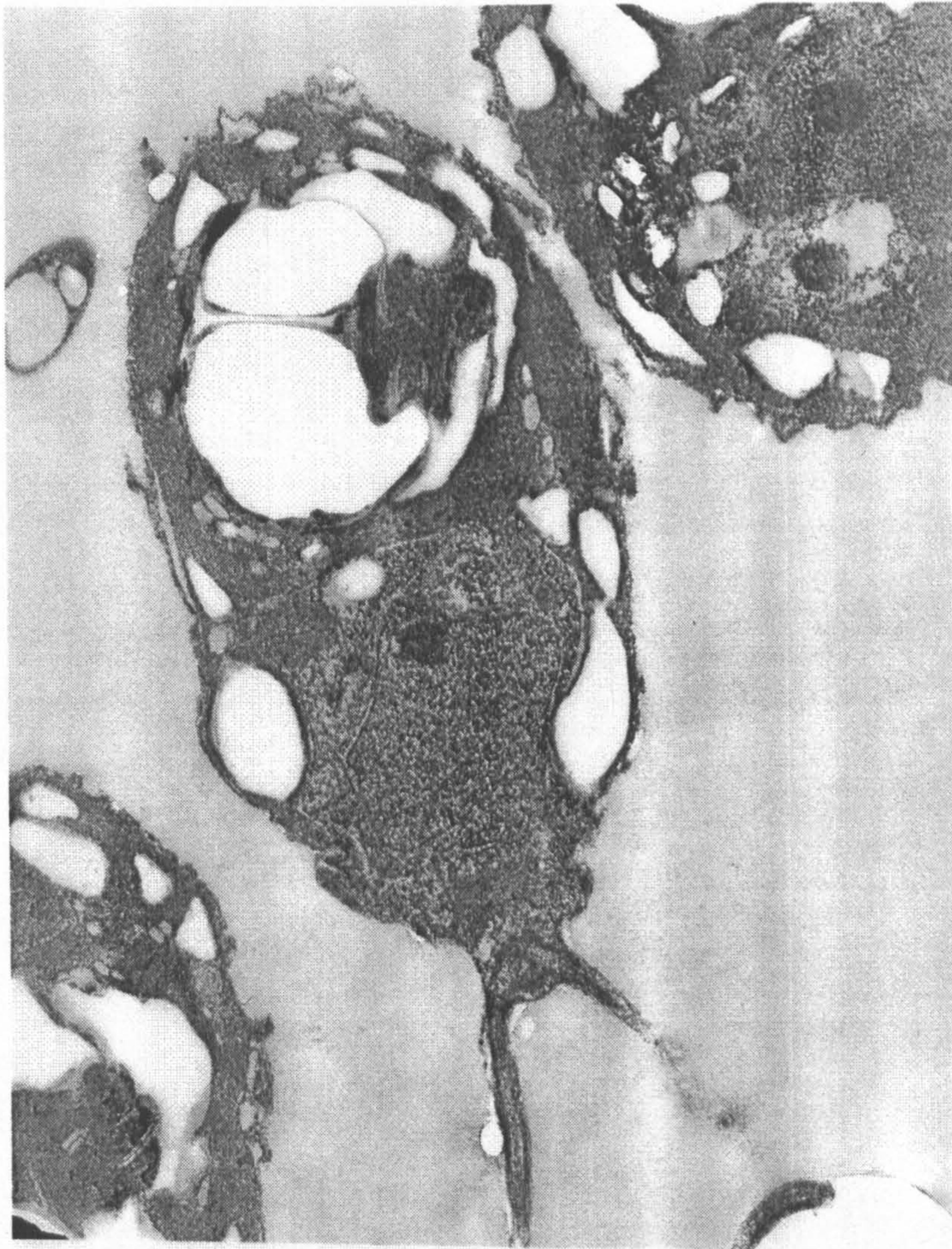


Figure 3.2: Electron micrograph for a single cell of *D. parva* grown in pH 7.5 medium. Magnification is at x 19600.



Figure 3.3: Electron micrograph for a single cell of *D. parva* grown in pH 5.5 medium. Magnification is at x 23000.

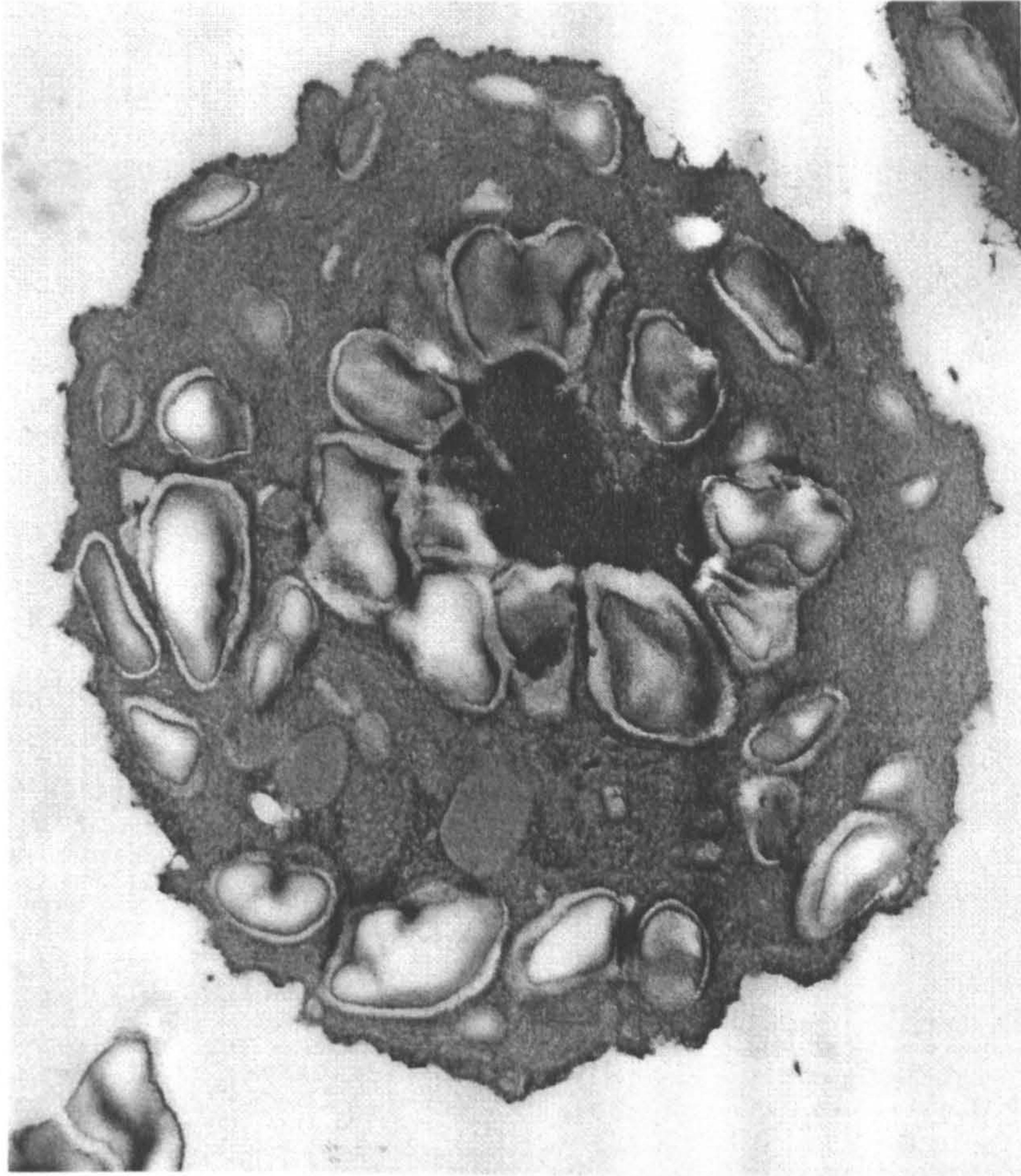


Figure 3.4: Electron micrograph for a single cell of *D. parva* grown in pH 9.0 medium. Magnification is at x 17800.

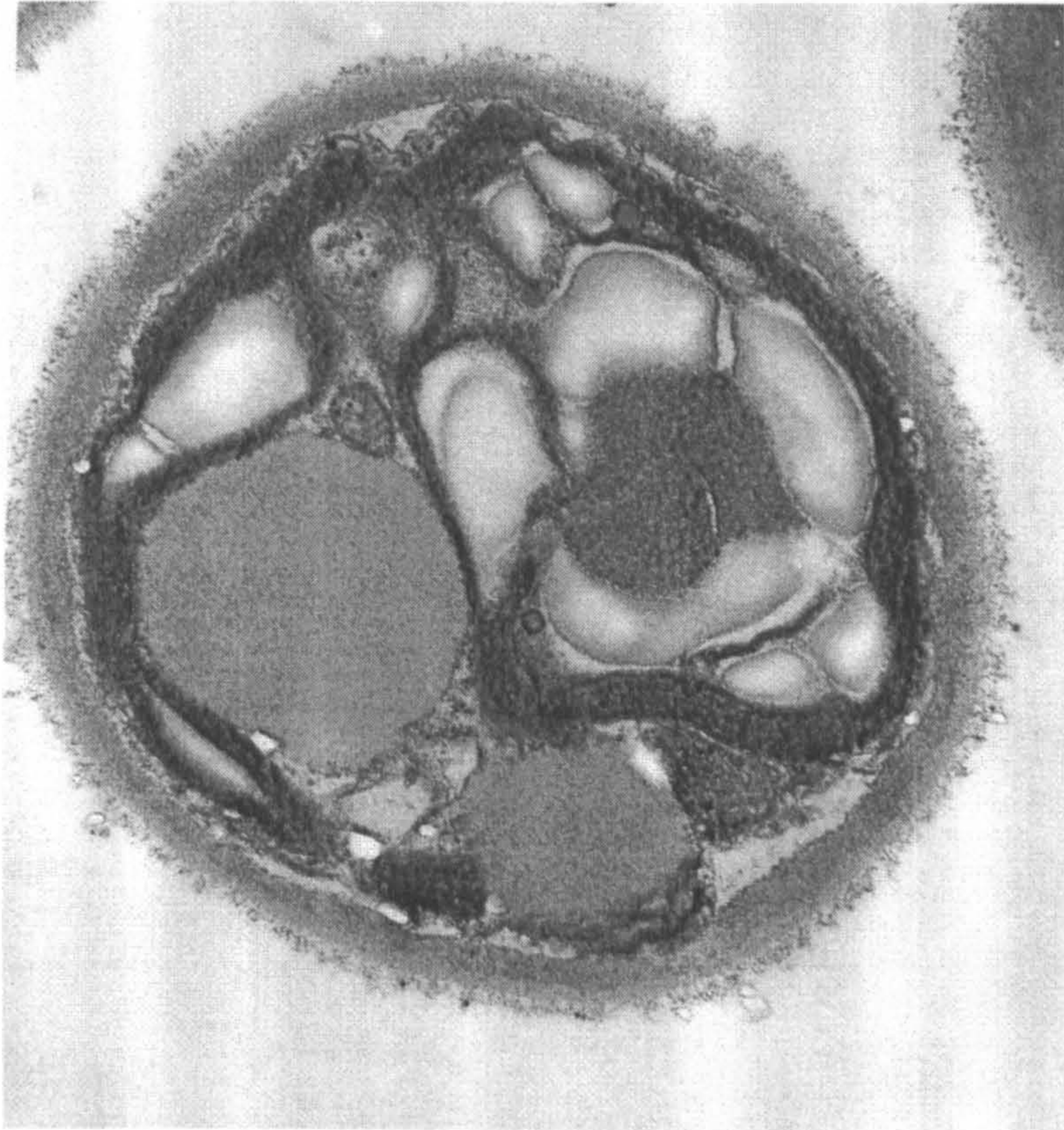


Figure 3.5: Electron micrograph for a single cell of *C. submarinum* grown in pH 5 medium. Magnification is at x 28000.

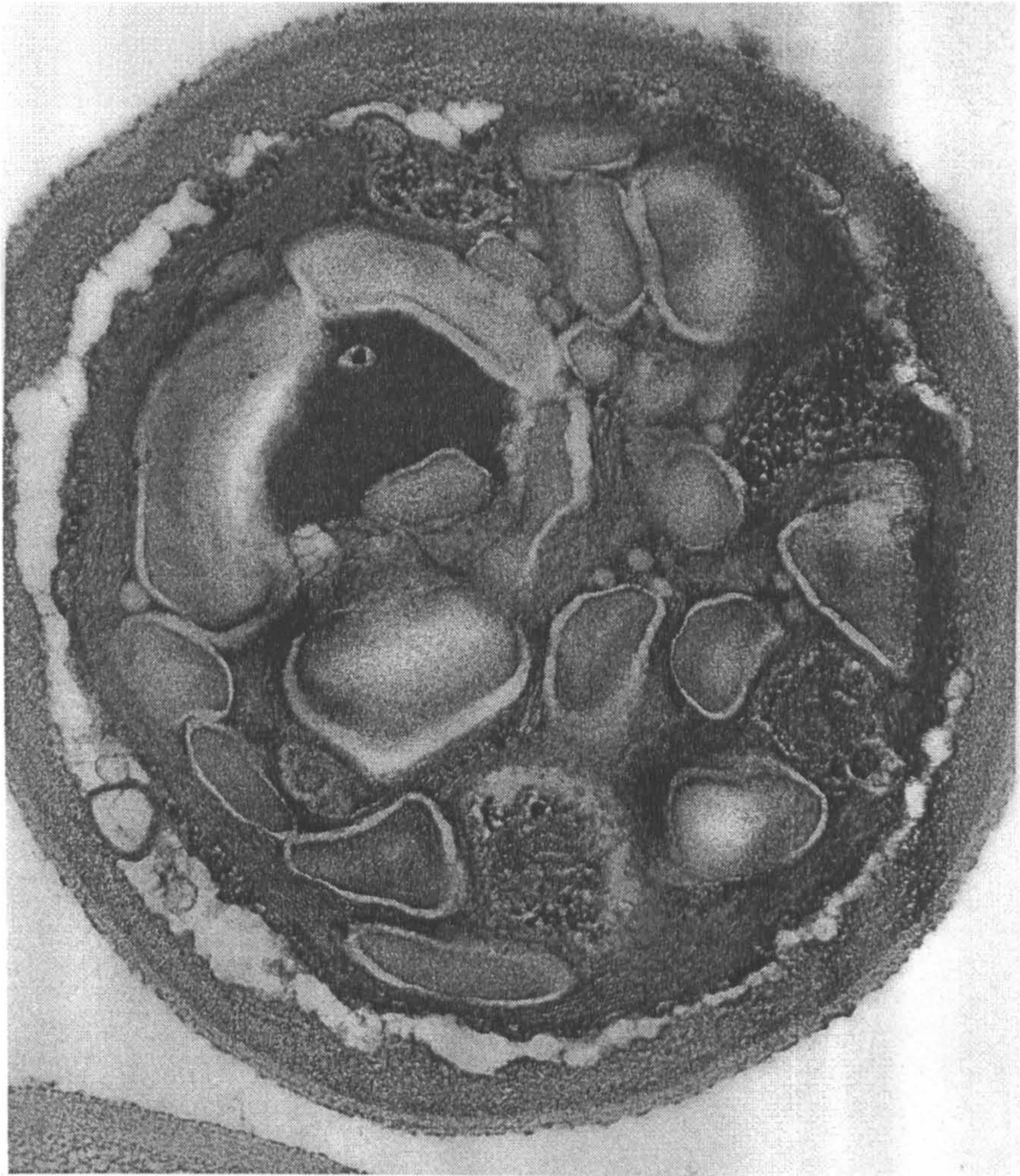


Figure 3.6: Electron micrograph for a single cell of *C. submarinum* grown in pH 5.5 medium. Magnification is at x 57000.

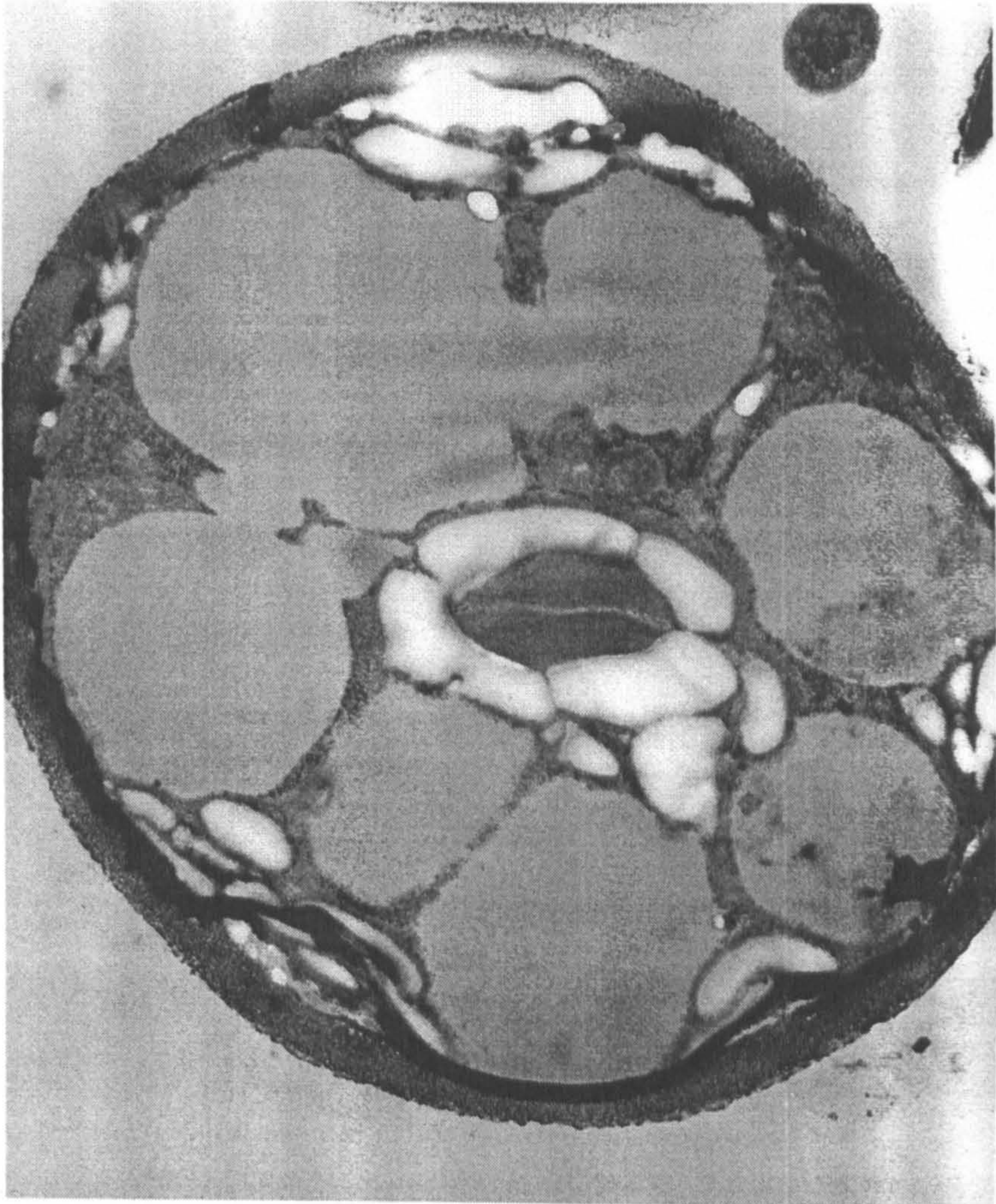


Figure 3.7: Electron micrograph for a single cell of *C. submarinum* grown in pH 9.0 medium. Magnification is at x 23000.

D. parva (Figures 3.2, 3.3, 3.4)

Figure 3.2 shows a *D. parva* cell grown in pH 7.5 medium. This electron micrograph is at a magnification of x 19600 and it can be seen that the flagella are still intact. The cell is clearly ovoid in shape unlike the cells in Figures 3.3 and 3.4. Figure 3.3 shows a *D. parva* cell grown in pH 5.5 media at a magnification of x 23000. This electron micrograph shows the distinctive cup - shaped chloroplast, nucleus and pyrenoid body. Figure 3.4 shows a pH 9.0-grown cell at a magnification of x 17800. The contents of the cell are not clear although it is possible to see the pyrenoid body and this cell appears to be very stressed. Again, as with the pH 5.5 stressed cell, the cell is round and not ovoid.

C. submarinum (Figures 3.5, 3.6, 3.7)

In all three electron micrographs of *C. submarinum* the thick cell wall is clearly visible. Lipid droplets are visible in cells adapted to all three pH levels, but are present in larger numbers in the cells adapted to pH 5.5 and 9.0.. Figure 3.5 is an electron micrograph of an individual *C. submarinum* cell grown in pH 7.5 medium, (magnification x 28000); the electron micrograph in Figure 3.6 is of a pH 5.5-grown *C. submarinum* cell (magnification x 57000) and the pH 9.0 grown *C. submarinum* cell is given in Figure 3.7 (magnification x 23000). All three cells look remarkably similar with their pyrenoid bodies and very thick intact cell wall although, the pH 9.0 cell appears more stressed

than the pH 5.5 grown cell. Figure 3.7 shows the split in the pyrenoid body clearly identifies the cells to be *C. submarinum* (Alvik, 1934).

3.2.5 Photosynthesis and Respiration Measurements

3.2.5.1 Cells adapted to a range of pH values

D. parva (Figure 3.8)

Highest rates of photosynthesis were found in pH 7.5 grown cells and lowest rates in pH 5.5 cells. The levels of photosynthesis between these samples were not significantly different at 5% level ($P = 0.206$). pH 9.0 grown cells produced the highest rates of respiration, similar to those produced by pH 7.5 grown cells ($P = 0.655$). The lowest rates of respiration were found in pH 5.5 grown cells although these were found not to be significantly different at 5% level when compared to the other two samples ($P = 0.111$ and $P = 0.370$ when compared to pH 7.5 and pH 9.0 adapted cells respectively).

The rates for photosynthesis and respiration were varied between the three pH levels examined although when statistically analysed they were not significantly different at 5% level. It can therefore be concluded from these results that growth at different levels of pH media had little effect on photosynthesis and respiration of *D. parva* cells adapted to pH 5.5, 7.5 or 9.0. This was also noted by Gimmler *et al.* (1988) who found photosynthesis only to be significantly reduced at extreme values (<5.5 and 9.0). pH 7.5

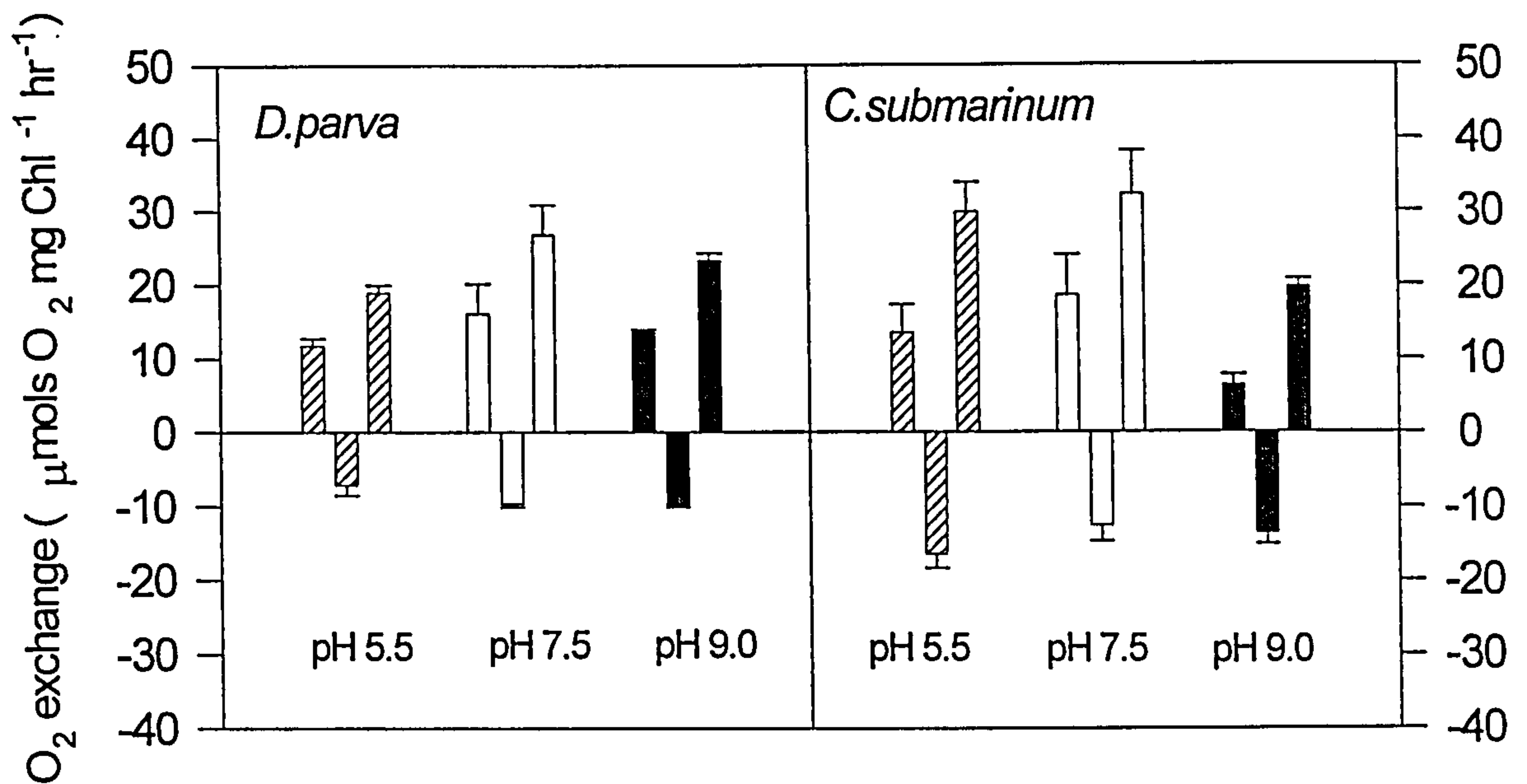


Figure 3.8: Effect of pH on Photosynthesis and Respiration of *D. parva* and *C. submarinum*. First column of each pH: oxygen evolution. Second column of each pH: respiration. Third column of each pH: total photosynthesis. Total photosynthesis is assumed to be equal to the rate of respiration plus oxygen evolution.

grown cells produced the highest levels of both photosynthesis and respiration, pH 5.5 grown cells showed the lowest rates. This information supports the data seen in the growth curves for *D. parva* (Figure 3.1) where highest growth levels were found in pH 7.5 adapted cells and lowest growth in pH 5.5 adapted cells.

C. submarinum (Figure 3.8)

As with *D. parva* the greatest rates of photosynthesis occurred in pH 7.5 grown cells. The oxygen evolution rates for these cells were not significantly higher than those found in pH 5.5 cells ($P = 0.872$). The oxygen evolution of pH 9.0 cells were seen to be over a third lower than those produced in pH 7.5 and pH 5.5 grown cells. Respiration rates for the pH 5.5 grown cells were significantly different from pH 7.5 and 9.0 grown cells. pH 5.5 grown cells produced the highest rates of respiration and pH 7.5 the least. pH 7.5 and 9.0 sample respiration rates were not significantly different ($P = 0.747$).

Overall, rates of photosynthesis and respiration in *C. submarinum* at pH 5.5 and 7.5 showed little effect of the different pH media. The photosynthesis rate for pH 9.0 grown cells was lower than the control of pH 7.5 grown cells ($P = 0.156$), but the respiration rate was not. Therefore, *C. submarinum* cells adapted to different pH levels appeared better able to cope with acidic pH than alkaline pH (Figure 3.8).

These data contradict the growth curve results for *C. submarinum* (Figure 3.1) where no differences in growth across the pH range were noted, although the differences in photosynthesis and respiration were not significant at 5% level.

3.2.5.2 Shock experiments

Cells grown in pH 7.5 medium were resuspended in fresh media set at pH 5.5, 7.5 and 9.0. The respiration and photosynthesis rates of all cells were measured immediately after the resuspension and again after three hours.

D. parva (Table 3.3)

Photosynthesis and respiration rates in all cells increased when measured after three hours when compared to the initial measurements. The initial low measurements could potentially be a combination of the shock effects of the centrifugation and resuspension in fresh media on the algae and the change in external pH. These can be differentiated by comparing the control (pH 7.5) with the shocked samples.

pH 7.5 grown *D. parva* cells resuspended in fresh pH 7.5 media produced the highest rates of photosynthesis and respiration when measured immediately and three hours after resuspension. These pH 7.5 cells showed significantly higher rates of photosynthesis with initial resuspension than pH 5.5 or 9.0 cells. When compared to the other pH grown cells P values were obtained ($P = 0.217$) for pH 5.5 grown cells and ($P = 0.204$) for pH 9.0 grown cells. Probability values for immediate photosynthesis rates and 3 hour photosynthesis rates indicated that there may have been a small effect of centrifugating and resuspending in fresh media shock (pH 7.5 = 0.732). An initial pH shock was also found in the cells resuspended in extreme pH media. This is apparent when comparing the immediate and three hour results (pH 5.5, $P = 0.488$; pH

9.0, $P = 0.237$). These results indicate that centrifugation and resuspension in fresh medium caused a slight but non-significant shock effect. This shock effect was small when compared to pH shock effects.

Rates of respiration measured for each of the three pH samples immediately after resuspension in the fresh media showed pH 7.5 cell levels to be highest. The results for pH 5.5 and 9.0 shocked samples were not significantly different ($P = 0.967$). Probability values of 0.342 and 0.334 were calculated when pH 5.5 and 9.0 cells were compared to pH 7.5 cells respectively. The results show that the immediate respiration measurements of pH 5.5 and 9.0 shocked cells were significantly lower than those measured for pH 7.5 cells, indicating that the pH shock was inhibiting metabolism.

After three hours in the fresh media both the photosynthesis and respiration levels for all cells increased. Photosynthesis increased 17.8%, 9.6% and 25.3% for pH 5.5, 7.5 and 9.0 cells respectively. Respiration of these cells also increased but at slightly lower levels (12.4%, 5.2% and 22.3% respectively). Highest rates of both photosynthesis and respiration were found in pH 7.5 control cells and the lowest in pH 5.5 shocked cells.

These data present the effect of pH shock on *D. parva* cells showing that a change in the pH of the medium has an immediate effect on the algae, decreasing oxygen evolution and oxygen uptake by the cells. It also shows that resuspension in fresh media also presents a small shock effect.

	$\mu\text{moles O}_2 \text{ exchange mg}^{-1} \text{ Chl h}^{-1}$			
	Immediately after shock		3 hrs after shock	
	Photosynthesis	Respiration	Photosynthesis	Respiration
pH 5.5	31.05 ± 2.56	10.30 ± 1.32	37.78 ± 6.91	11.76 ± 1.18
pH 7.5	42.61 ± 6.80	14.46 ± 3.23	47.14 ± 8.36	15.26 ± 2.96
pH 9.0	31.72 ± 2.83	10.41 ± 1.68	42.49 ± 6.63	13.40 ± 1.95

Table 3.3: Shock Effects of pH on Photosynthesis and Respiration in *D. parva*.

Total photosynthesis is assumed to be equal to the oxygen uptake in the dark plus oxygen evolution.

For all samples pH 7.5 cells showed the highest levels of error, this may be due to the large number of samples studied on different days. The absolute values vary from day to day. Control measurements were taken frequently between the pH stressed samples to ensure the equipment was functioning correctly, resulting in higher number of control samples measured.

C. submarinum (Table 3.4)

Highest rates of photosynthesis were found in pH 7.5 grown cells resuspended in pH 7.5 media. This was true for the samples measured immediately and three hours after resuspension in fresh media.

Immediately after resuspension in fresh medium, cells exposed to all three pH levels showed similar rates of photosynthesis. After three hours resuspension in the fresh media the photosynthetic rates for all three pH samples dropped. The largest decrease was seen in pH 5.5 shocked cells (62.0 %). pH 9.0 shocked cells reduced their photosynthetic rate by one fifth (20.3 %) and pH 7.5 control cells reduced their rates by a very small amount (7.4 %).

Respiration rates at all three pH levels were maintained at constant levels after the three hour resuspension period. Respiration rates for pH 9.0 shocked cells remained the same over the three hour period ($P = 0.765$) while pH 7.5 cells increased their rates marginally (14.0 %) and pH 5.5 cells decreased their rates slightly (14.2 %).

The drop in photosynthesis levels after three hours, in pH 5.5 and 9.0 cells, was probably a result of the change in external pH. The pH 7.5 (control) cells showed no difference in photosynthesis over the three hour period ($P = 0.896$) thus, it can be concluded that resuspension in fresh media does not cause a significant shock effect. The delay in the reduction in photosynthetic rate may be due to the thick cell wall in *C. submarinum*. This thick cell wall may be able to initially protect the cell from a sudden

	$\mu\text{moles O}_2 \text{ exchange mg}^{-1} \text{ Chl h}^{-1}$			
	Immediately after shock		3 hrs after shock	
	Photosynthesis	Respiration	Photosynthesis	Respiration
pH 5.5	31.90 ± 10.10	9.29 ± 2.23	12.11 ± 1.48	7.97 ± 1.76
pH 7.5	33.91 ± 10.95	9.71 ± 1.03	31.41 ± 11.57	11.07 ± 3.63
pH 9.0	30.45 ± 9.75	10.53 ± 1.67	24.28 ± 7.21	9.37 ± 2.73

Table 3.4: Shock effects of pH on Photosynthesis and Respiration in *C. submarinum*. Total photosynthesis is assumed to be equal to the oxygen uptake in the dark plus oxygen evolution.

change in external pH but after three hours exposure to the pH change the cells show signs of pH stress.

3.2.5.3 Effect of Inhibitors

Cells were taken from cultures grown at pH 5.5, 7.5 and 9.0 and rates of photosynthesis and respiration measured in the presence of CCCP and monensin. Ethanol was added to the control, because CCCP is dissolved in ethanol. Monensin was dissolved in water,

and it was assumed that the small volumes of water used had no effect on the algae. It should be noted that in these experiments CCCP was not being used as uncoupler of ATP synthesis (a role it does not appear to play in unicellular green algae), but instead it was simply being used to inhibit both photosynthesis and respiration (see section 1.5.2).

D. parva - CCCP (Table 3.5)

Ethanol appeared to have a minor effect on photosynthesis and respiration (data not shown). The sensitivity of the algae to CCCP changed with the pH of the external medium. CCCP was seen to have the greatest effect on pH 5.5 grown cells, stopping photosynthesis and significantly reducing respiration at the lowest levels of inhibitor studied (5 μ M). pH 9.0 grown *D. parva* cells showed great resistance to CCCP for both photosynthesis and respiration. At the highest levels of CCCP used (100 μ M) these cells were able to photosynthesize and respire at relatively high levels (at least 33 % of control).

pH 7.5 grown cells also showed resistance to this inhibitor. Photosynthesis was only stopped with the highest level of CCCP (100 μ M) with respiration being reduced to about one third of the control level.

The ineffectiveness of CCCP at alkaline pH values has been noted by various authors working on bacterial respiration (e.g. Tokuda and Unemoto, 1983; MacLeod *et al.*, 1988). However, MacLeod *et al.* (1988) suggested that the reduced inhibitory action of CCCP at alkaline pH was due to a physical chemistry effect i.e. as the pH is increased

above the pK for CCCP (6.1), the proportion of the undissociated form is reduced which decreases the ability of CCCP to transport H^+ across the membrane (section 1.5.2).

It has already been mentioned that CCCP does not cause uncoupling in algae, and was instead used here as a metabolic poison in the current experiments. However, the decrease in undissociated CCCP molecules would mean that the rate of entry into the *D. parva* cells was reduced. Therefore, this may be the basis of the lack of inhibition by CCCP at pH 9.0.

D. parva - Monensin (Table 3.6)

In all pH levels tested there was a gradual reduction in both photosynthesis and respiration rates. Even at the highest levels of monensin studied (100 μ M) photosynthesis continued in pH 7.5 cells albeit at a reduced level, and respiration was largely unaffected. There appeared to be little difference in the effect of the inhibitor on the cells grown at different pH levels, although there was some evidence that pH 9.0 grown cells were more sensitive to high concentrations of monensin.

Inhibitor	$\mu\text{moles O}_2 \text{ exchange mg}^{-1} \text{ Chl h}^{-1}$					
	pH 5.5		pH 7.5		pH 9.0	
	Photosynth.	Respiration	Photosynth.	Respiration	Photosynth.	Respiration
Control + ETOH	24.0 ± 3.24	8.67 ± 0.87	38.4 ± 4.50	7.2	26.5 ± 1.04	5.63 ± 0.78
5 μM CCCP	-0.2	3.8	30.3 ± 2.48	7.8	-	-
10 μM CCCP	-	-	17.3 ± 2.26	7.6	-	-
25 μM CCCP	-0.3	2.2	1.95 ± 0.31	3.9	25.6 ± 3.54	5.31 ± 0.22
50 μM CCCP	-0.5	1.3	0.65 ± 3.25	3.7	17.9 ± 1.19	4.36 ± 0.31
75 μM CCCP	-	-	0.35	3.6	11.3	3.13
100 μM CCCP	-	-	-1.3	2.6	9.17 ± 4.13	3.96 ± 0.59

Table 3.5: Effect of CCCP on Photosynthesis and Respiration in *D. parva*. The rate of photosynthesis was equal to dark oxygen uptake plus oxygen evolution in the light.

Inhibitor	$\mu\text{moles O}_2 \text{ exchange mg}^{-1} \text{ Chl h}^{-1}$					
	pH 5.5		pH 7.5		pH 9.0	
	Photosynth.	Respiration	Photosynth.	Respiration	Photosynth.	Respiration
Control + ETOH	24.0 ± 3.24	8.67 ± 0.87	38.4 ± 4.50	7.17 ± 0.61	26.5 ± 1.04	5.63 ± 0.78
5 μM MON.	-	-	-	-	-	-
10 μM MON.	17.5	15.5	43.6	9.1	-	-
25 μM MON.	13.4	9.99	13.8	7.12	16.5 ± 2.72	4.46 ± 0.30
50 μM MON.	8.9	6.0	8.01	5.9	13.8 ± 4.28	4.38 ± 0.30
75 μM MON.	7.0	7.0	10.6 ± 1.74	6.72 ± 0.35	4.79 ± 0.95	4.79 ± 0.45
100 μM MON.	-	-	9.10 ± 1.62	6.99 ± 0.62	-	-

Table 3.6: Effect of Monensin on Photosynthesis and Respiration in *D. parva*. The rate of photosynthesis was equal to dark oxygen uptake plus oxygen evolution in the light.

C. submarinum - CCCP and Monensin (Table 3.7)

The inhibitor studies were only possible with *C. submarinum* at pH 7.5. At extremes of pH the cell density required to give measurable readings in the oxygen electrode was very high, and photoinhibition may have occurred. At high chlorophyll content the oxygen evolution of the cells could have been measured, but the results produced would have been inaccurate and hence impossible to measure any effects of the inhibitor present.

It was established that the ethanol added had little or no effect on the rates of photosynthesis and respiration (data not shown). CCCP was found at high concentrations to have a detrimental effect on both photosynthesis and respiration of *C. submarinum* cells grown at pH 7.5. At concentrations up to 25 μM CCCP there appeared to be little consequence of the inhibitor, with photosynthesis and respiration rates being reduced only very slightly. At 50 μM CCCP photosynthesis was reduced to a greater extent, again there was little reduction in respiration rate. At 75 μM CCCP photosynthesis was almost completely inhibited and rates of respiration greatly reduced. These low levels of photosynthesis and respiration were maintained for 100 μM CCCP.

A comparison of Tables 3.5 and 3.7 shows that pH 7.5 grown *C. submarinum* cells were more resistant to CCCP than pH 7.5 grown *D. parva* cells. This may indicate that CCCP was excluded more effectively from *C. submarinum* cells, because of the thick cell wall.

Inhibitor	$\mu\text{moles O}_2 \text{ exchange mg}^{-1} \text{ Chl h}^{-1}$			
	CCCP		Monensin	
	Photosynth.	Respiration	Photosynth.	Respiration
Control + ETOH	29.53 ± 3.12	13.10 ± 0.29	29.53 ± 3.12	13.10 ± 0.29
5 μM	29.75	12.75	-	-
25 μM	25.50	12.75	-	-
50 μM	15.41 ± 2.05	10.63 ± 1.33	23.73 ± 4.32	11.33 ± 2.09
75 μM	1.77 ± 0.29	4.60 ± 0.29	18.42 ± 3.63	9.92 ± 1.90
100 μM	1.06 ± 0.50	4.25 ± 0.50	18.77 ± 4.37	10.63 ± 1.73

Table 3.7: Effect of CCCP and Monensin on Photosynthesis and Respiration in *C. submarinum* cells grown at pH 7.5. The rate of photosynthesis was equal to dark oxygen uptake plus oxygen evolution in the light.

Monensin reduced both photosynthesis and respiration in *C. submarinum* at high concentrations (Table 3.7). At 50 μM monensin both photosynthesis and respiration were at nearly control levels. At 75 μM and 100 μM monensin, oxygen evolution and uptake was reduced, but the cells appeared to be able to function quite normally. A comparison of Tables 3.6 and 3.7 shows that *C. submarinum* is again more resistant to this inhibitor than *D. parva*, presumably because of the exclusion of monensin by the cell wall of *C. submarinum*.

3.2.6 Cell Volume Determination in Different pH Media.

3.2.6.1 Micrometric measurements

Cells of *D. parva* and *C. submarinum* were grown in medium with a pH of 5.5, 7.5 and 9.0. Measurements of the long and short axes of *D. parva* and single axis of *C. submarinum* allowed the cell volumes to be calculated (section 2.11.1).

D. parva (Figure 3.9)

Cells of *D. parva* grown over a range of pH media were found to have different volumes. This was concluded to be a direct result of pH stress. pH 7.5 cells were assumed to be under no external pH stress and were also assumed to be of "normal" volume.

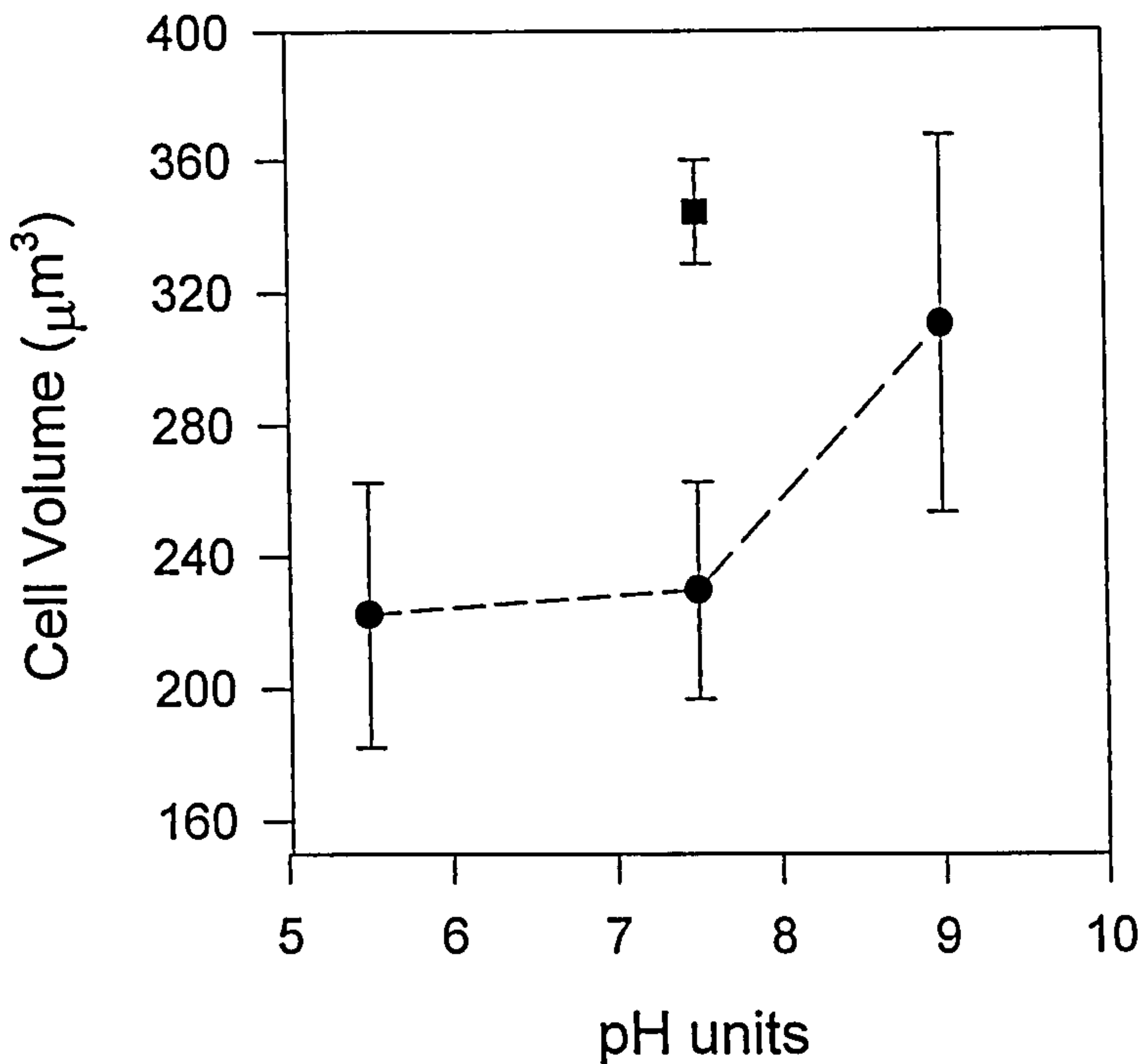


Figure 3.9: Micrometric Measurements of Cell Volumes of *D. parva* and *C. submarinum* - Effect of pH. ● = *D. parva*; ■ = *C. submarinum*.

pH 9.0 grown cells have been found to be substantially, but not significantly (at the 5% level), larger than the pH 7.5 cells ($P = 0.245$) (Figure 3.9 and 3.10). The increase in cell volume may be considered to be a self defence mechanism of the algae to the extreme pH of the medium. Encysting occurred, as separation, after cell division, took place less rapidly than in normal cells. The inner-most cells were protected from the external stress as they were surrounded by the older cells and therefore not exposed to

the external stress. Cell volumes at pH 5.5 showed very little difference to volumes of pH 7.5 grown cells, suggesting that *D. parva* is better able to cope at acidic pH than alkaline pH.

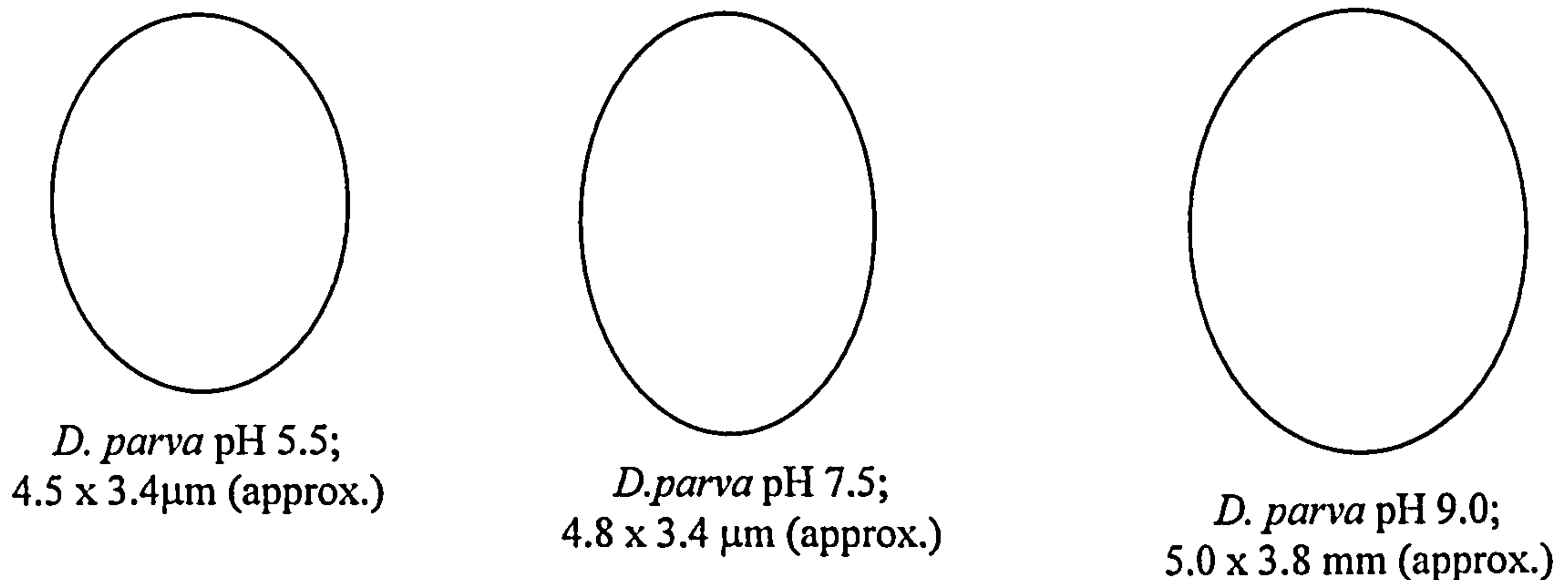


Figure 3.10: Diagrammatic representation of *D. parva* cells size once exposed to external pH stress. Scale 1 μm = 1 cm (approx.). These diagrams are not completely accurate in size but they do represent the effect of pH stress on cell size, and shape.

From Figure 3.9 it is evident that cells grown at extremes of pH show greater sample to sample variation than those grown at pH 7.5.

C. submarinum (Figure 3.9)

At pH 5.5, it was not possible to accurately measure *C. submarinum* cells. Individual cells were indistinguishable and only clumps of algae visible. pH 9.0-grown *C. submarinum* cells were discovered to be encysting. Many smaller motile cells were

also observed. These smaller zoospores released from the encysting cells were found to be six times more abundant than the larger cells. Therefore, it was not possible to determine cell volume in pH 9.0 grown cells. However, it was possible to measure the volumes of pH 7.5 cells. They were calculated to be much larger than *D. parva* cells grown at the same pH (Figure 3.9), with an average diameter of 4-6 μm . Blackwell *et al.* (1991) noted that vegetative *C. submarinum* pH 7.5 grown cells varied between spherical cells 10-15 μm in log phase and cells 5-7 x 8-11 μm in stationary phase culture. Therefore, the measurements made during this project using the direct micrometric method showed smaller cell volumes than those found by Blackwell and Gilmour (1991b). The reason for this discrepancy is not clear. However, Blackwell and Gilmour (1991b) also noted that the tendency for *C. submarinum* cells to clump during normal growth invalidated direct methods of measurement of cell size and cell number per unit volume. Also, the micrometric measurements here would take into account the thick cell wall whereas indirect measurements would not.

3.2.6.2 Silicone oil measurements

The silicone oil technique allows the determination of volume of all cells grown over a wide pH range (section 2.11.2).

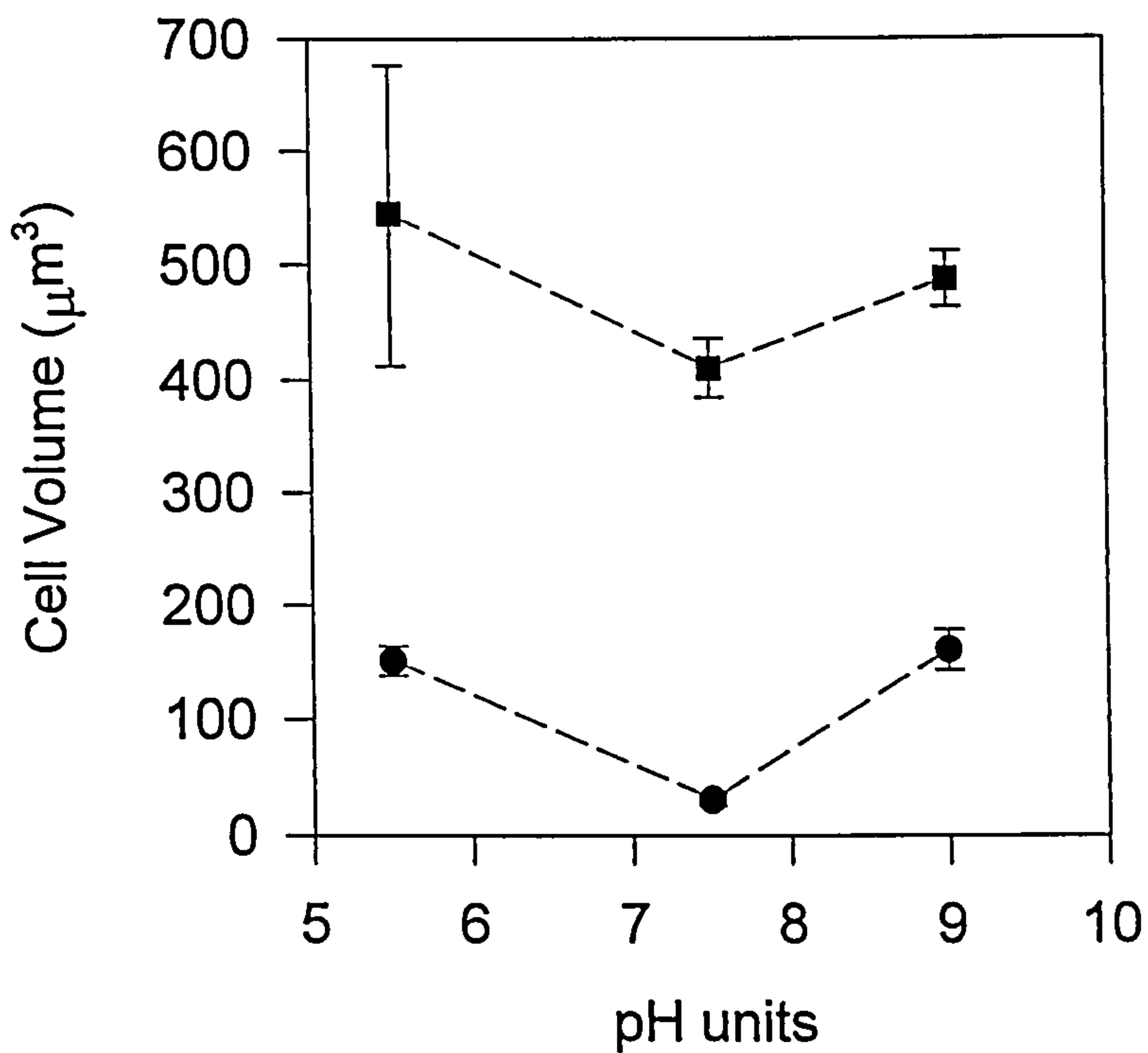


Figure 3.11: Cell Volume Measurements for *D. parva* and *C. submarinum* using Silicone Oil Technique. ● = *D. parva*; ■ = *C. submarinum*.

D. parva (Figure 3.11)

D. parva cells grown in pH 7.5 medium were smaller than those cells grown in media with a pH of 5.5 and 9.0. Cells grown at pH 5.5 and 9.0 were found to have very similar volumes. The similarity in these larger cell volumes may be a response of the cells to external pH stress. The stressed cells may not have separated after dividing,

resulting in clumps of cells being measured and not single cells, or the onset of cell division may have been delayed by the unfavourable pH conditions.

A comparison of Figures 3.9 and 3.11 shows a large discrepancy in the cell volumes measured directly and by the silicone oil technique. At all pH levels, the cell volumes determined were less than those determined by the micrometric methods although at pH 5.5 this difference was not significant at 5% (pH 5.5 $P = 0.236$; pH 7.5, $P = 3.04 \times 10^{-6}$; pH 9.0, $P = 0.0096$). This casts doubt on the suitability of the silicone oil method and suggests that ^3H -dextran may not remain outside of the *D. parva* cells (see section 2.11.1).

C. submarinum (Figure 3.11)

Using the silicone oil method, *C. submarinum* cells were found to have volumes of over three times those found for *D. parva* cells using the same method, the largest difference in size being in the pH 7.5 grown cells. *C. submarinum* cells grown in pH 5.5 and 9.0 medium were larger than those grown at pH 7.5 (Figure 3.12). This may be a cellular response to extremes of external pH. Alternatively, it is possible that the silicone oil technique encounters problems distinguishing individual *C. submarinum* cells from clumps, therefore, the volume measurements may be of the stressed clumps of cells rather than single cells. Hence, the cell volumes calculated for *C. submarinum* using the silicone oil were higher than expected and the error large, particularly for pH 5.5 grown cells. It should be noted that the cell volume measurements for *C. submarinum*

cells grown in pH 7.5 media for both methods agree pretty closely (micrometric measurements = $344.16 \pm 15.73 \mu\text{m}^3$: silicone oil measurements = $410.0 \pm 25.5 \mu\text{m}^3$).

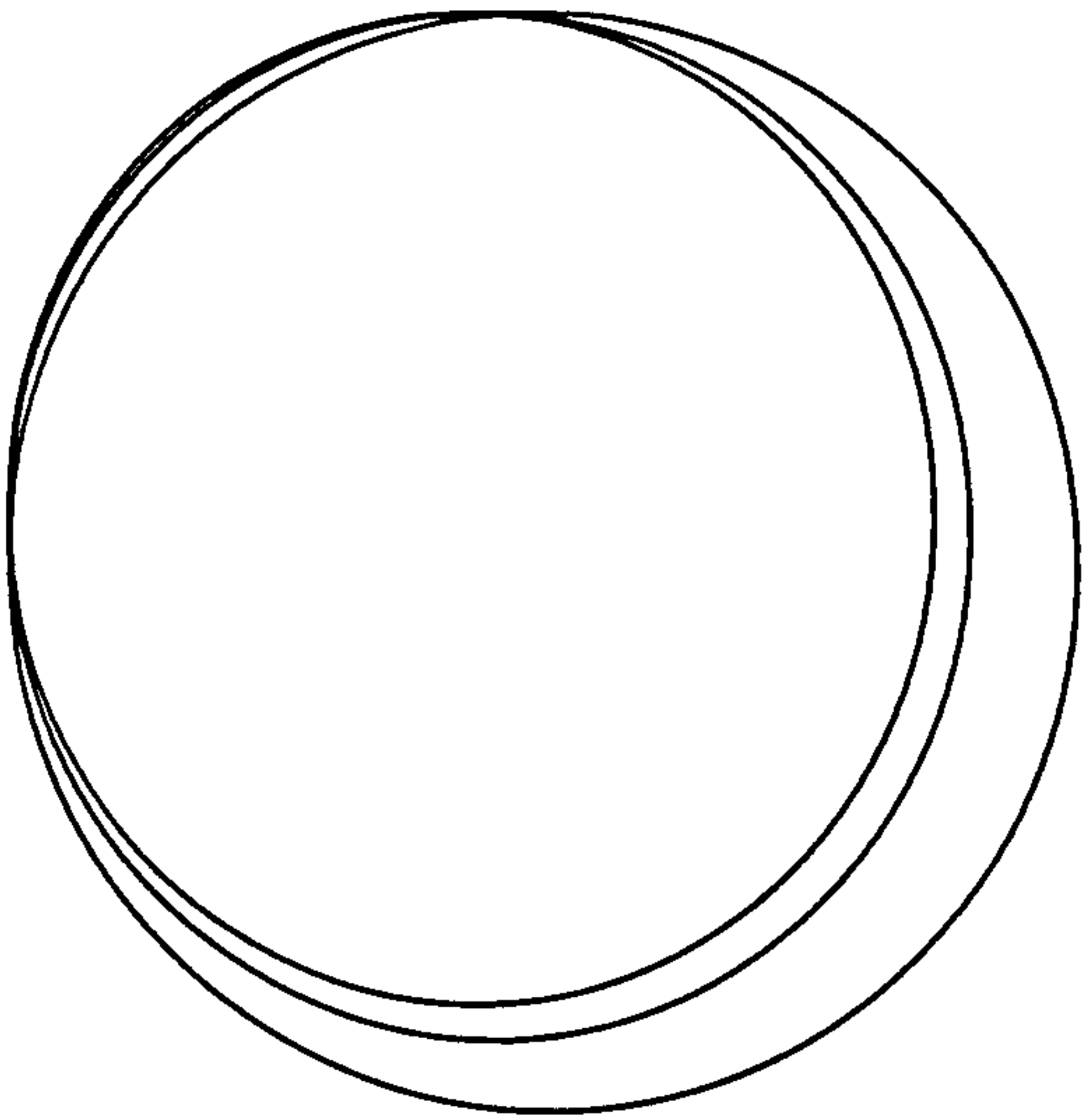


Figure 3.12: This figure demonstrates clearly the difference in cell size measured by silicone oil method for *C. submarinum* grown in different pH media. The largest circle represents a cell grown in pH 5.5, the middle sized circle represents a cell grown in pH 9.0 and the smallest circle represents a cell grown in pH 7.5. These diagrams are not completely accurate in size but they do represent the effect of pH stress on cell size.

External stress on algal cells has been found by many groups to have a profound effect on cell size. Changes in cell volume in *D. salina* were found by Cowan *et al.* (1995), by monitoring the chemical shift in P_i , to occur complementarily with changes in compartmental pH. Weiss and Pick (1996) established that *D. acidophila* survived for

several days but did not divide when pH stressed at pH 7.0. Golldack *et al.* (1995) noted that salt stressed *D. parva* grew more slowly than those at optimum salt concentrations and the cells divided less frequently and had bigger cell volumes. In contrast to this Cowan *et al.* (1995) found exposure to salt stress caused cell shrinkage. There is no doubt that external stress on algal cells does effect cell volume. These changes in cell size may be a result of reduced cell division, encysting cells, cell clumping or a combination of these factors.

There may be a problem in the use of the isotope ^3H -dextran for the measurements of intracellular volume. Figure 4.2b, section 4.2.1, shows a time course of dead cells and alive cells with dextran. The dextran should not be taken up by the cells though this data showed this to be happening. This might explain the discrepancies in the measurement of cell volume in the *D. parva* cells.

3.2.7 Estimation of cell numbers at a range of pH values

Samples of each alga, over a range of chlorophyll contents ($5\text{-}60\ \mu\text{g Chl ml}^{-1}$), were prepared and the cell number counted.

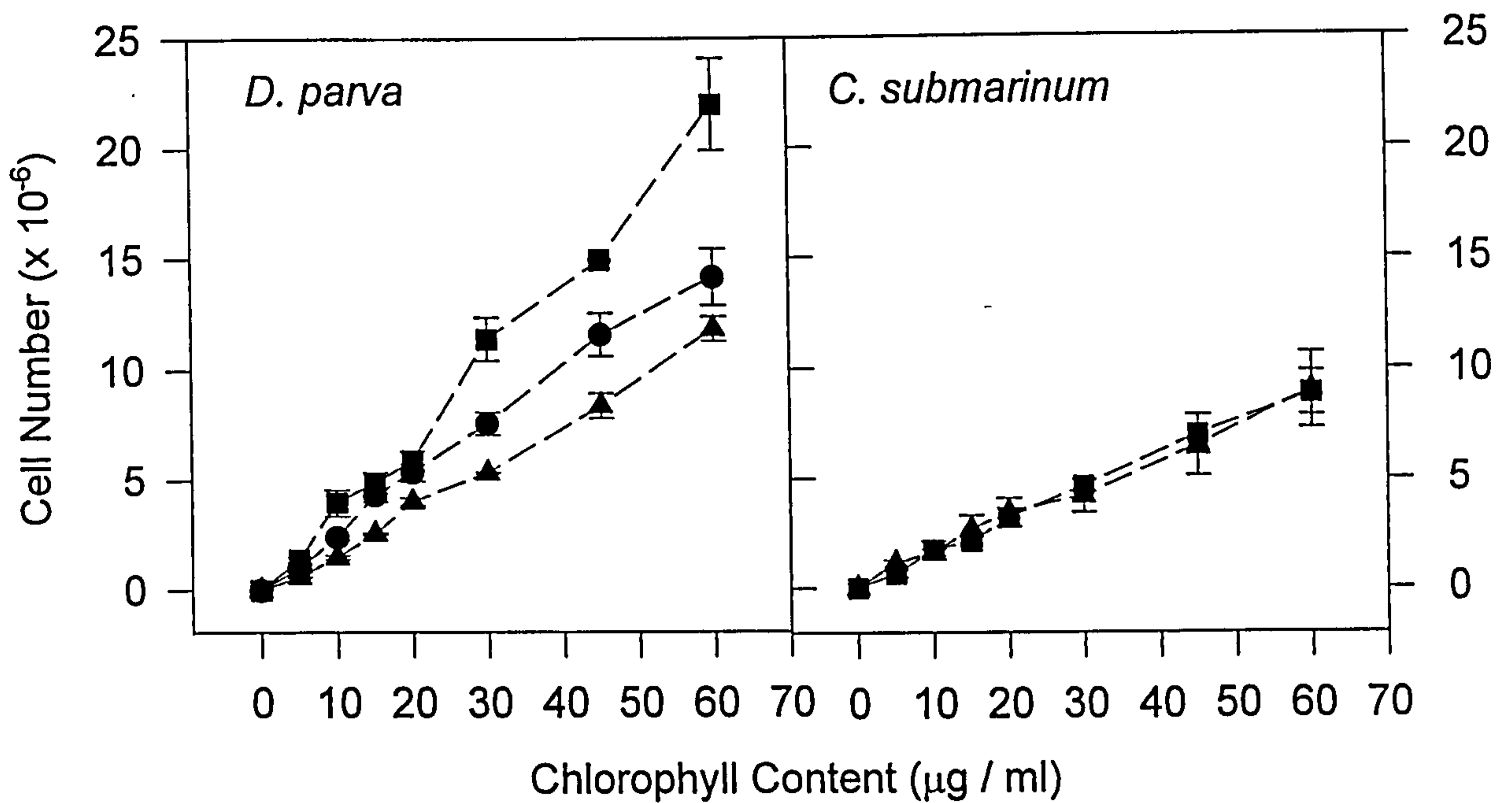


Figure 3.13: Cell Number versus Chlorophyll Content for *D. parva* and *C. submarinum*, grown at pH 5.5 (●), 7.5 (■), 9.0 (▲).

D. parva (Figure 3.13)

The results produced for *D. parva* showed a large difference in cell number per unit chlorophyll over the pH range. However, differences in cell number were not significant at 5% level and the greatest difference found was between the pH 7.5 grown cells and the pH 9.0 grown cells ($P = 0.195$). The cell number was reduced at extremes of pH for all chlorophyll levels.

These lower counts were probably a result of the cells not dividing as a response to the pH stress and also limitations in growth rate of individual cells. These differences should be taken into account when chlorophyll content is used as an estimate of cell number.

C. submarinum (Figure 3.13)

At pH 5.5, it was not possible to accurately count *C. submarinum* as individual cells, only clumps of algae were visible. pH 9.0 grown *C. submarinum* cells were found to be encysting. Many smaller motile cells were also observed (Section 3.2.6.1).

The results for *C. submarinum* cells grown at pH 7.5 and pH 9.0 showed some differences, but it can be concluded that these differences were not significant ($P = 0.97$) and that cell number per unit chlorophyll measurements for *C. submarinum* were uniform at pH 7.5 and 9.0.

3.2.8 Protein Determination of *D. parva* and *C. submarinum* cells.

After growth at each of the external pH values the cells of both algae were concentrated to a range of chlorophyll contents and the protein measured using the Bradford method (section 2.6).

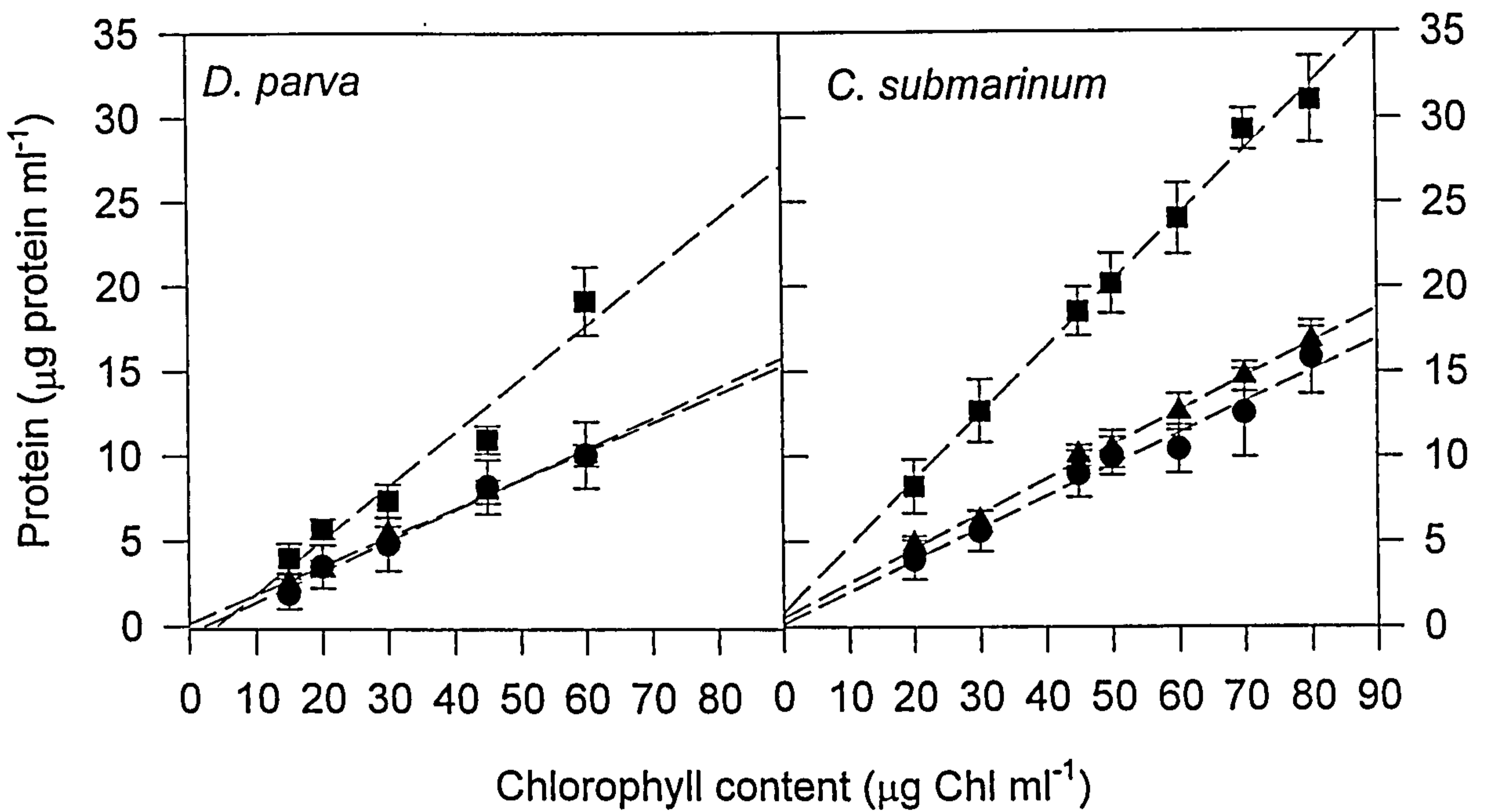


Figure 3.14: Protein Content versus Chlorophyll Content for *D. parva* and *C. submarinum* grown at pH 5.0 (▲), 7.5 (●), 9.0(■). Best fit lines were not put through zero as this would be an assumption that when there was no chlorophyll there was no protein.

D. parva (Figure 3.14)

Plotting chlorophyll concentrations against protein illustrated that at pH 9.0 there was an elevated level of protein present in the cells. The increased level of protein in pH 9.0-grown cells can be directly related to the increased volume at this pH.

pH 5.5 and 7.5 grown algae had lower protein content values when compared to pH 9.0 grown algal cells but similar values to each other ($P = 0.94$). For all algae the protein levels increased as the chlorophyll levels increased in a linear fashion.

C. submarinum (Figure 3.14)

The protein measurements taken for *C. submarinum* produced similar results to those produced for *D. parva*. pH 9.0 grown algae showed the highest levels of protein. pH 5.5 and 7.5 grown cells produced lower levels of protein. The results for *C. submarinum* cells at pH 5.5 and 7.5 were more readily distinguishable than the results produced for *D. parva* ($P = 0.61$). pH 7.5-grown algae were seen to have the lowest protein levels.

As with *D. parva* the differences in protein could be directly related to the number of algal cells present and/or cell volume.

3.2.8.1 Protein Determination per Algal Cell

The number of algal cells present changed depending on pH of the growth media therefore the protein determinations made in section 3.2.8 were converted to protein per cell (Table 3.8).

pH	<i>D. parva</i>			<i>C. submarinum</i>		
	Protein per Cell (x10 ⁻¹²)	Cell Volume (μm ³)	Protein per Cell Volume (μg protein ml ⁻¹ volume)	Protein per Cell (x10 ⁻¹²)	Cell Volume (μm ³)	Protein per Cell Volume (μg protein ml ⁻¹ volume)
5.5	0.691	150.8	0.0046	0.818	544.18	0.0015
7.5	0.469	30.73	0.0153	0.732	410.0	0.0018
9.0	1.461	160.35	0.0091	1.553	487.38	0.0032

Table 3.8: Table Showing Protein for *D. parva* and *C. submarinum* μm³ grown at pH 5.5, 7.5 and 9.0. Calculated from protein present per cell and cell volume at different pH values.

The results show clearly that for both *D. parva* and *C. submarinum* the highest levels of protein per cell were found in pH 9.0 grown cells and the lowest in pH 7.5 grown cells.

3.2.8.2 Protein versus cell volume

Protein per cell volume was also calculated using cell volumes from the silicone oil technique as this method produced values for all cells (Table 3.8). The results show that for *C. submarinum* the highest protein level per cell volume was found in pH 9.0 grown cells and there was little difference between protein per cell volume for pH 7.5 or

pH 5.5 grown *C. submarinum* cells. For *D. parva*, protein per cell is maximal at pH 7.5, but this is due to the very low cell volume measured for pH 7.5 grown *D. parva* cells.

Cell protein composition has been directly linked with stress. It can be seen quite clearly from Figure 3.15 that the amount of protein in both *D. parva* and *C. submarinum* cells changed considerably under the stress of extreme external pH. Satoh and Shiraiwa (1996) showed that in *Chlorella regularis* a particular polypeptide was induced at pH 5.5, but not at pH 8.0 and another induced at both pH 5.5 and pH 9.0. Golldack *et al.* (1995) showed that with salt stress *D. parva* showed a number of modifications in protein synthesis, the majority of these changes occurred within a few hours following the stress, some proteins of *D. parva* increased with increased salinities, others decreased.

3.2.9 Glycerol Determination of *D. parva* and *C. submarinum* cells

Glycerol was determined using the acetylacetone method (section 2.7). Glycerol concentrations for cells grown at all pH levels were determined.

D. parva (Table 3.9)

The highest level of glycerol found in *D. parva* cells was for pH 9.0 grown cells. These levels were double those found in the pH 5.5 and 7.5 grown cells. The glycerol concentrations found in pH 5.5 and 7.5 cells were very similar to each other ($P = 0.92$).

pH	Glycerol ($\mu\text{moles glycerol per cell} * 10^6$)	
	<i>D. parva</i>	<i>C. submarinum</i>
5.5	1.38 ± 0.08	0.37 ± 0.03
7.5	1.41 ± 0.05	0.17 ± 0.06
9.0	2.97 ± 0.22	0.27 ± 0.03

Table 3.9: Effect of pH on Glycerol Concentrations in *D. parva* and *C. submarinum*.

Glycerol is the compatible solute produced by *Dunaliella* species during times of salt stress (section 1.3). The high levels of glycerol produced for pH 9.0 cells is a surprising result. It is very unlikely that the glycerol production at pH 9.0 is a direct result of the high pH of the media. The most likely explanation is an error in cell number determination at pH 9.0. The levels of glycerol production in these cell were very low when compared to salt stressed *D. parva* cells. The results below are those of S. Powtongsook (PhD Student, Sheffield University) and show the vast difference in glycerol produced in medium of different salinity.

pH	μg glycerol per μg Chl
7.5; 1.5 M NaCl	8.42
7.5; 3.5 M NaCl	24.4

The salt stressed 3.5 M cells had production of glycerol 2 times larger than the 1.5 M NaCl grown cells. In comparison the pH 9.0 grown cells only had an increased glycerol production 2 times when compared to the pH 7.5 grown cells (Table 3.9).

C. submarinum (Table 3.9)

The glycerol levels found in *C. submarinum* cells were much lower than those found in the *D. parva* cells because *C. submarinum* was grown at 0.25 M NaCl and *D. parva* was grown at 1.5 M NaCl. pH 5.5-grown cells produced the highest levels of glycerol and pH 7.5-grown cells the lowest. The levels of glycerol produced were too low to be able to draw any firm conclusions about the effect of different external pH values. Blackwell and Gilmour (1991b) showed that glycerol was the major internal solute for salt stressed *C. submarinum* cells and that the internal concentrations of glycerol were very strongly correlated with external salinity. However, it is not known whether *C. submarinum* also uses the glycerol cycle for production/removal of glycerol in the same way as *D. parva* (section 1.3).

3.2.10 *C. submarinum* growth after resuspension in fresh media

C. submarinum cells were resuspended in fresh media with different pH values. Growth of the cells was monitored by counting cell numbers (Table 3.10). All cultures started with the same cell number as they all came from the same initial culture. After 5 days the control cells (pH 7.5) showed the highest cell number (although there were large variations from day to day). After a 3 day lag phase, growth started in pH 9.0. No growth took place in pH 5.5 media during the five days of the experiment.

The number of “normal” single cells in each culture was compared to the number of larger, encysting cells present (Figure 3.15). Before the samples were resuspended in new media the cells present were over 90% single. Over six days a steady decrease in the percentage of single cells in all cultures was seen. In pH 7.5 cultures the number of single cells stayed above 65%, unlike pH 5.5 cells which dropped to below 20% and pH 9.0 cells which reached almost 50%. At pH 5.5 after five days many of the encysting cells were seen to be moving on the spot. It appeared that they had lost their outer membrane. Many small motile cells were seen also, the cells were starting to clump as well as encyst. This shows that the number of motile and non-motile cells varied over the five day growth period and this related to pH stress.

Time (Days)	Cell number ($\times 10^7$)		
	pH 5.5	pH 7.5	pH 9.0
0	1.4	1.4	1.4
1	1.14	1.83	1.32
2	1.28	1.55	1.30
3	1.03	1.76	1.39
4	1.22	1.40	1.99
5	1.33	2.60	2.11

Table 3.10: Growth (Cell numbers) of *C. submarinum* in Different pH Media.

3.3 Conclusions

It can be seen from this chapter that both species of algae are able to cope with changes in external pH stress. *C. submarinum*, possessing a cell wall, has an advantage over *D. parva* and this is evident from the growth curves (assuming that the precipitation of the media had no effect on the growth of the alga) (Figure 3.1).

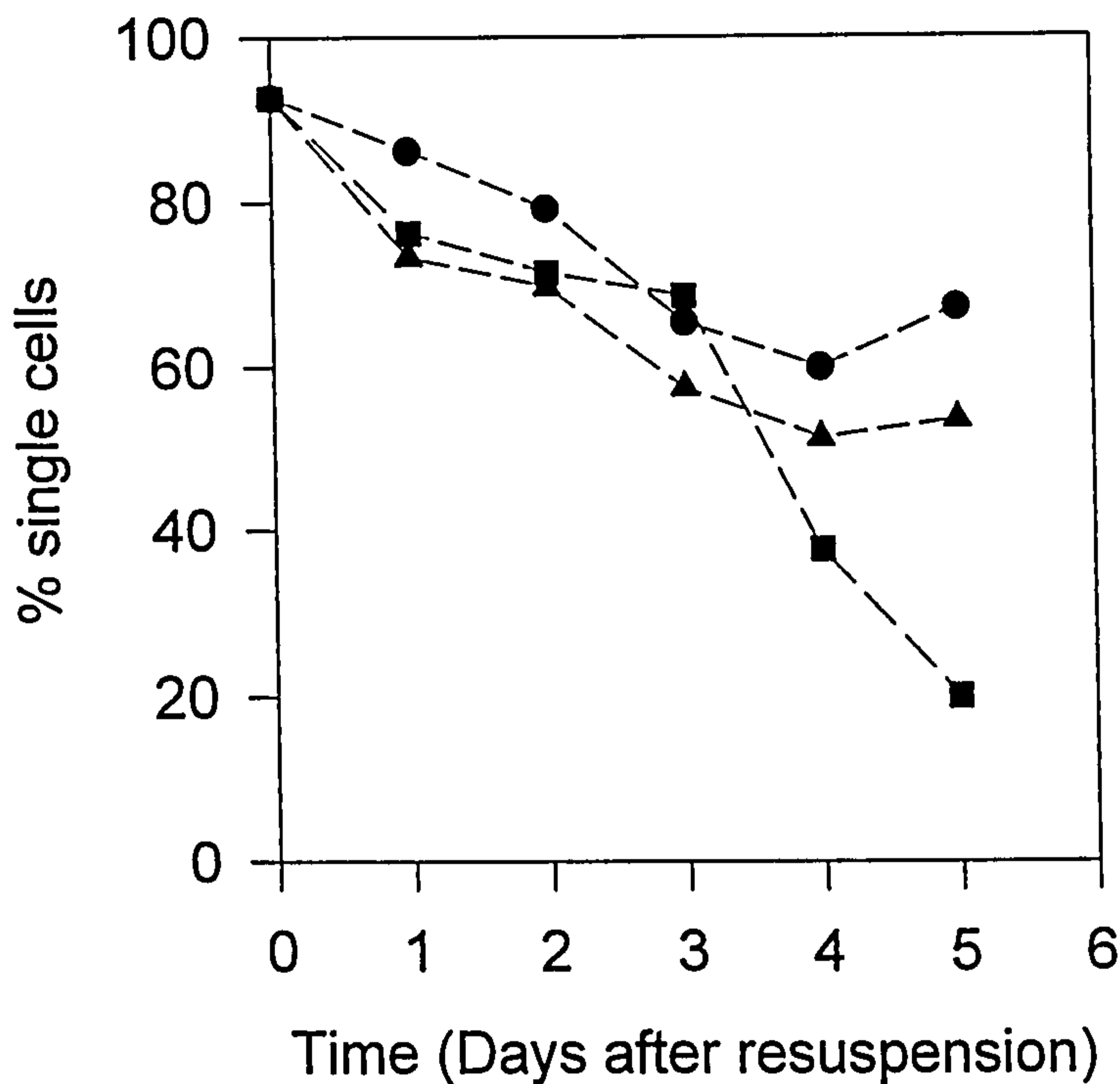


Figure 3.15: Percentage of Single *C. submarinum* Cells, Effect of pH Over Time.
 pH 7.5 (●), 5.5 (■), 9.0 (▲).

Media at extremes of pH appear to have detrimental effects on the growth of *D. parva* cells. The pH stressed cells produced moderate rates of growth, but, when compared to the growth of the alga at pH 7.5, these were greatly reduced. Extremes of external pH appeared to have little effect on growth of *C. submarinum* over the twenty one day time period. The electron micrographs of both algae support this data with the *C. submarinum* cells showing little effect of the external medium and *D. parva* becoming visually stressed at both acidic and alkaline external pH (Figures 3.2-3.7).

In the pH adapted cells photosynthesis and respiration rates were reduced in both algae (Figure 3.8). In *D. parva* the lowest rates were found in pH 5.5 grown cells. The reduction in rates found in *C. submarinum* were small and algae adapted to an acidic external pH were found to function at a more “normal” level than those adapted to an alkaline pH.

The samples resuspended in fresh media of different pH levels produced contrasting results (Table 3.3). The initial rates for *D. parva* were low, after three hours they increased. The opposite occurred in *C. submarinum* cells. The delayed reduction in the rates in *C. submarinum* cells was probably due to the presence of a thick cell wall. In *D. parva* cells the resuspension in fresh medium, regardless of pH shock, had a small but negative effect on the cell metabolism. These cells were quick to adapt to their new pH media and able to photosynthesise and respire at near normal rates within three hours. In both algae, pH 5.5 cells showed more reduced levels of photosynthesis and respiration than pH 9.0 shocked cells.

D. parva was found to be more sensitive to inhibitors than *C. submarinum* (Tables 3.5, 3.6 and 3.7), this was probably a direct result of *C. submarinum* possessing a thick cell wall. *D. parva* was more sensitive to CCCP than monensin as was *C. submarinum*. Cells grown at acidic pH were found to be more susceptible to CCCP than those grown at alkaline pH. This was explained in section 3.2.5.3, *D. parva*. Monensin appeared to show little difference in its ability to inhibit across the pH range although maybe there

was some role for a Na^+/H^+ antiporter in maintaining internal pH *D. parva* pH 9.0 grown cells as at this pH photosynthesis and respiration were slightly reduced..

There are various methods used to measure cell volume, but we have used silicone oil, an indirect method and a direct micrometric method. Zmiri *et al.* (1984) measured cell volume by three different methods, two direct methods and one indirect method, electrical sizing. They found that the direct methods produced similar results, these results being consistently larger than those obtained by electrical sizing. It was concluded that the significant difference was due to the cell membrane having an unusually low electrical resistance relative to other cell membranes (Zmiri *et al.* 1984). Determination of cell volume using silicone oil technique involves the radioisotopes $^3\text{H}_2\text{O}$ and ^3H -dextran. The $^3\text{H}_2\text{O}$ is evenly distributed throughout the pellet, whereas the ^3H -Dextran, which has a molecular weight of 70,000, is only found in the spaces between the cells in the pellet (Ginzburg, 1969). The extracellular space and intracellular volume can be calculated using these markers (Rottenberg 1979, 1989). It appears from the results presented here that the dextran has been broken down and is being incorporated into the cell thus producing incorrect results (section 4.2.1).

Direct and indirect measurements found cell volume of both algae to increase when grown in pH stressed conditions (Figures 3.9 and 3.10). The indirect (silicone oil) technique produced larger volumes for *C. submarinum* cells and smaller volumes for *D. parva* when compared to micrometric measurements. Another method for cell volume calculation would be required before any direct conclusions could be drawn as it was seen that both micrometric and Silicone Oil produced values at the low end of the scale

for *D. parva* (Preisig, 1992). It has been established by both methods that the cell size does increase with external stress and this increase in cell volume is probably a result of slower division of cells. Cell number in stressed cells of *D. parva* also decreased as a result of the reduced cell division, while the cell number in *C. submarinum* was maintained.

Protein in the algal cells increased with external stress (Figure 3.14). For both algae pH 9.0 grown cells produced the highest levels. It has been well established that cell protein increases with external stress but it would be interesting to establish if these elevated levels of protein are a result of particular stress related proteins or a general increase in intracellular proteins. Specific stress-related proteins have been established in *Dunaliella* spp. for external stress (Ginzburg *et al.*, 1990; Fisher *et al.*, 1997) and other proteins have been found to increase with external stress (Kampen *et al.*, 1995).

Bioenergetics of pH tolerance in
Dunaliella parva and *Chlorococcum*
submarinum.

CHAPTER FOUR

4.1 Introduction

It is essential for algal cells to maintain a stable internal balance when exposed to an external stress. It is therefore very important to study the internal pH and membrane potential of the cell in relation to changes in external pH. The maintenance of a neutral internal pH allows the cell to function normally. This maintenance of a near neutral internal pH at extremes of external pH maybe aided by changes in the membrane potential.

Membrane potential can be defined as the potential difference that would be measured between two identical reference electrodes positioned on opposite sides of a membrane. It is a property of the whole system and is independent of the spatial location of the electrodes (Rottenberg, 1979). Under normal circumstances membrane potential is negative due to fixed charges in the cell membrane. The most reliable method for measuring membrane potential is the use of micro-electrodes across the membrane, but microalgal cells are too small and thus an indirect method must be used. $^3\text{H-TPP}^+$ (tetraphenylphosphonium), can be used for measuring membrane potential, because this positively charged ion is distributed across the membrane according to the membrane potential.

Internal pH can be determined by an indirect method based on weak acid and weak base probes. There are two forms of acid or base probes - charged and uncharged. The uncharged form enters the cell and equilibrium is reached depending on the cell

pH (Figure 4.1). ^{14}C -methylamine, a weak base, is used for external pH values 7.5-10.0 and ^{14}C -benzoic acid, a weak acid, for external pH values 4.5-7.5.

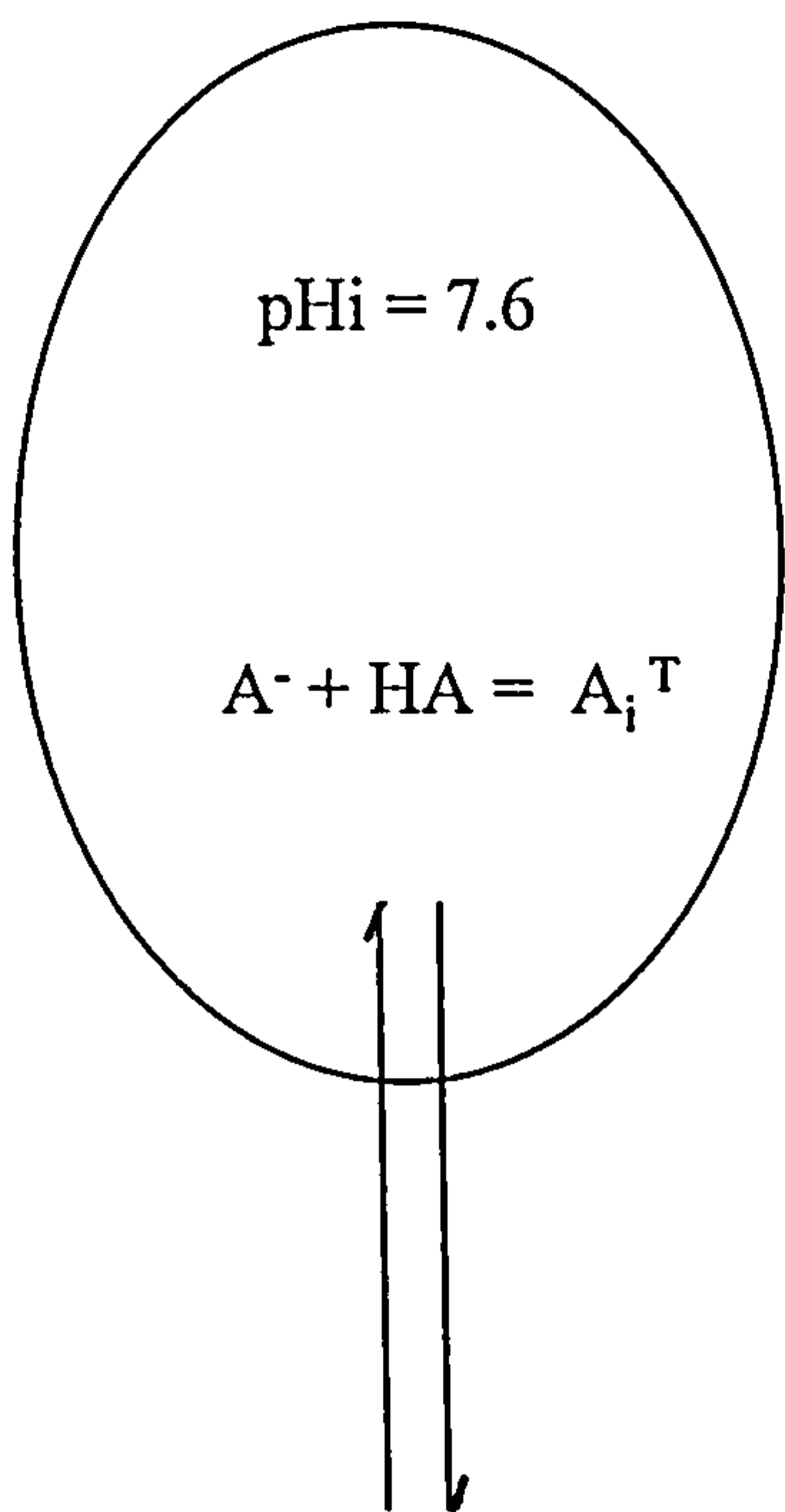
4.2 Results and Discussion

4.2.1 Isotope uptake in Dead and Live Cells

Gimmler and Greenway (1983) produced data indicating that in *Chlorella emersonii* TPP^+ was not suitable as a probe for the measurement of electrical potentials. They established that a large uptake of ^{14}C - TPP^+ took place even after the cells had been killed and there was no significant difference in the incorporation of TPP^+ into living cells compared with dead cells. The most likely explanation is that TPP^+ was adsorbed on to the cell wall of the alga. It is also essential that the probe was not metabolised but taken up by diffusion (Gimmler *et al.*, 1989) and hence the initial experiments with *D. parva* and *C. submarinum* looked at isotope uptake in algal cells in dead and live states. All five isotopes, tritium, ^{14}C -benzoic acid, ^{14}C -methylamine, ^3H -dextran and ^3H - TPP^+ were examined for this problem. These isotopes were added to the algal cells and after a period of incubation the cells were separated from the medium by silicone oil centrifugation. To determine the length of time the algae should be incubated with each isotope, time courses were determined (Section 4.2.2) and the time taken to reach equilibrium was noted for each isotope. Time courses were also carried out for samples of dead algae to ensure that the isotopes did not adhere to the cell surface. The inhibitor carbonylcyamide m-chlorophenyl hydrazone

Weak Acid pK = 5

pH_o = 6



Weak Base pK = 9

pH_o = 10

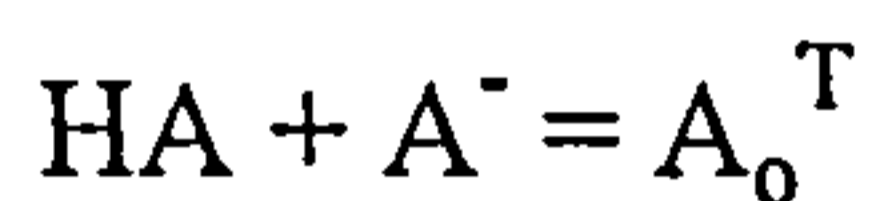
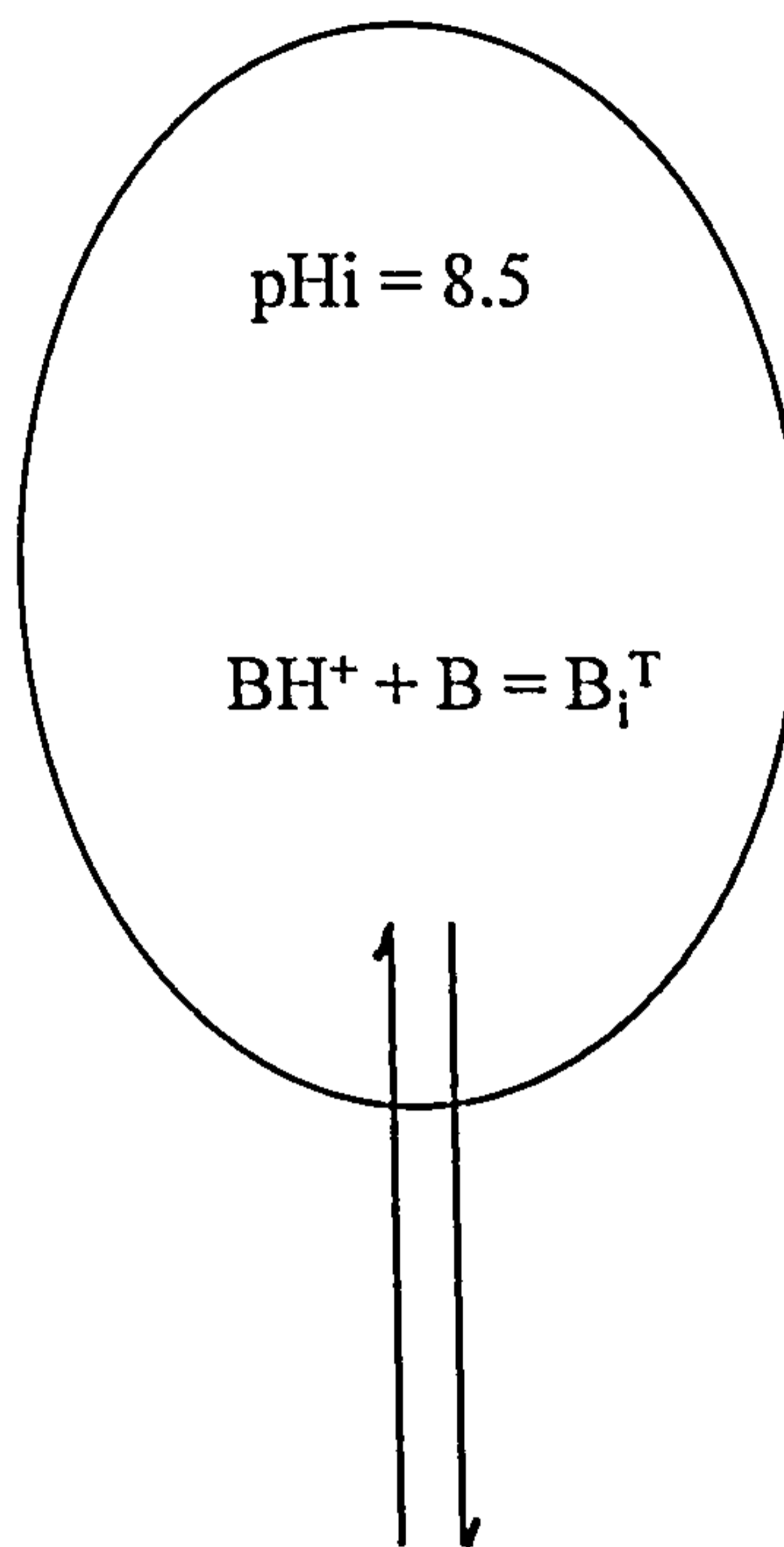


Figure 4.1: Diagram of the equilibrium reached with a weak acid or a weak base. The concentration of HA is the same on both sides of the membrane, while A⁻ is concentrated in the alkaline compartment. The concentration of B is the same on both sides of the membrane, while BH⁺ is concentrated in the acidic compartment. At the pK of the weak acid or base the charged and uncharged forms are found in roughly equal amounts in the external medium.

(CCCP) was added to the algal cells (100 μM for *D. parva* and 200 μM for *C. submarinum*) and left for an hour before the isotope was added. The cells were assumed to be dead when they no longer photosynthesised (Table 4.1).

Figures 4.2 (a, b) compare the uptake of the isotopes in dead cells over time for *D. parva* and *C. submarinum*. The measurement of interest was the activity found in the pellet after silicone oil centrifugation. In *D. parva* cells (Figures 4.2 a, b) the levels of radioactivity found in the pellets was significantly higher in all of the live cells except tritium. Tritium and dextran levels should not differ between living and dead cells since they are distributed according to size. Water penetrates into the cell and the dextran does not. These results imply that dextran was being taken up by the cells and was therefore not suitable as an extracellular marker. This may have been the result of the dextran being broken down over time into smaller compounds that were therefore more likely to be incorporated into the cell. The extracellular volume measurement (ECV) calculated using dextran played a minor role in the calculation of membrane potential and internal pH and thus any errors in calculating the in extracellular volume had little or no effect on the final results for membrane potential and internal pH. This can be demonstrated by altering the ECV to extreme values:

ICV = 0.647		
ECV = 0.263	ECV = 0.430	ECV = 0.647
$\Delta \Psi = -49.73$	$\Delta \Psi = -47.6$	$\Delta \Psi = -44.87$
Internal pH = 6.25	Internal pH = 6.25	Internal pH = 6.25

This clearly shows that when the ECV was at extreme values the $\Delta \Psi$ changed only slightly and the internal pH did not change at all.

Inhibitor \ treatment	<i>D. parva</i>		<i>C. submarinum</i>	
	Photosynthesis	Respiration	Photosynthesis	Respiration
pH 7.5 control	22.9	7.25	37.38	11.38
control + 20 μ l Ethanol	21.38	6.11	31.96	11.38
100 μ M CCCP (Immed.)	0.77	3.82	n/a	n/a
100 μ M CCCP (1hr)	0.00	1.15	n/a	n/a
200 μ M CCCP (Immed.)	n/a	n/a	6.50	5.42
200 μ M CCCP (1hr)	n/a	n/a	0.00	1.63

Table 4.1: Effect of the inhibitor CCCP on photosynthesis and respiration in *D. parva* and *C. submarinum* immediately after inhibitor addition and 3 hours later.

n/a = not applicable-samples were not taken.

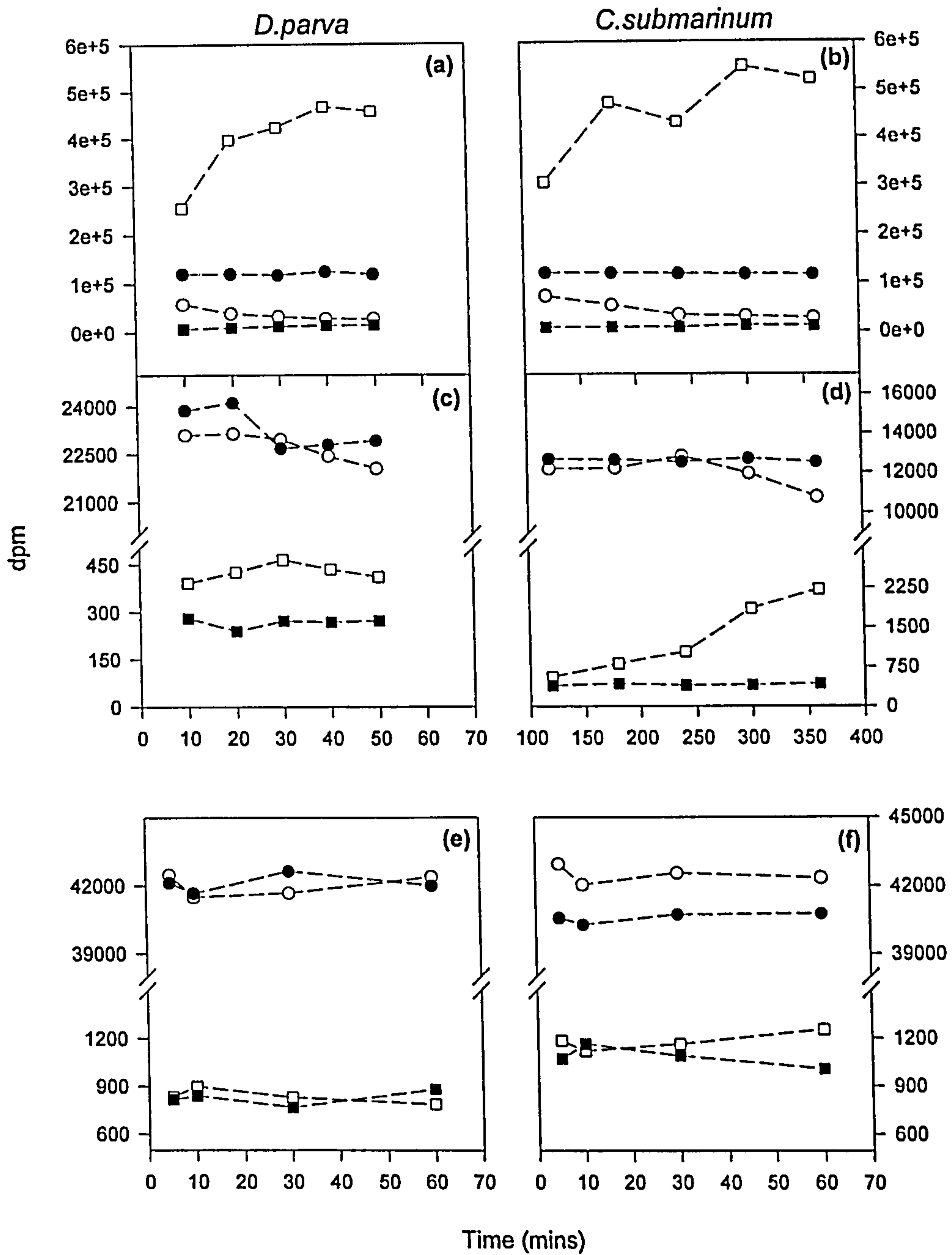


Figure 4.2a: ^{14}C -Methylamine (a,b), ^{14}C -Benzoic Acid (c,d) and $^3\text{H}_2\text{O}$ (e,f) uptake in Dead and Live *D. parva* and *C. submarinum* Cells Over Time. ● = dpm in supernatant of dead cells; ○ = dpm in supernatant of live cells; ■ = dpm in pellet of dead cells; □ = dpm in pellet of pellet of live cells.

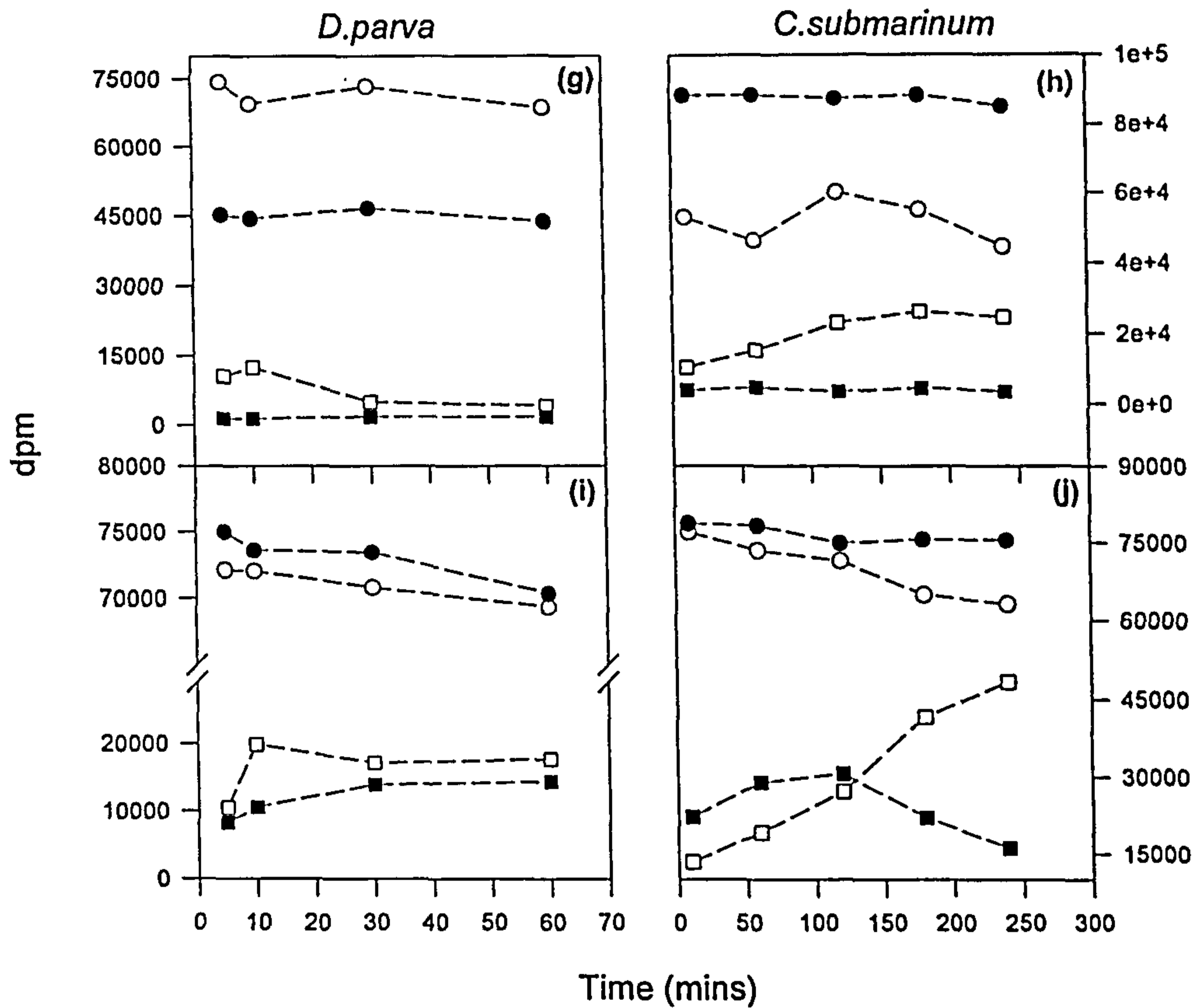


Figure 4.2b: ³H-Dextran (g,h) and ³H-TPP⁺ (i,j) uptake in Dead and Live *D. parva* and *C. submarinum* Cells Over Time. ● = dpm in supernatant of dead cells; ○ = dpm in supernatant of live cells; ■ = dpm in pellet of dead cells; □ = dpm in pellet of pellet of live cells.

Similar results were found for *C. submarinum* (Figures 4.2 a, b) although the levels of ³H₂O found in the pellets of living cells were very slightly higher than those in the pellets of dead cells (P = 0.0639).

From these results it was possible to conclude that all the isotopes used except ^3H -dextran were suitable probes for *D. parva* and *C. submarinum* in the silicone oil experiments. It has already been demonstrated that the problems with ^3H -dextran uptake by the cells do not affect the determination of membrane potential and internal pH values.

4.2.2 Time courses of Radiolabelled Isotopes

An essential prerequisite for the applicability of TPP^+ to estimate the membrane potential was that the TPP^+ concentration in the cells reached a maximum value with time (Gimmler and Greenway, 1983). This prerequisite was applied to all isotopes used in the present study.

Figures 4.2 (a, b) show time courses for *D. parva* and *C. submarinum*. The period of time taken for saturation to be reached was noted and the results are summarised in Table 4.2.

The length of time for the isotopes to reach a maximum level in pellets of *C. submarinum* cells was longer than for *D. parva* cells, this was probably a result of *C. submarinum* possessing a cell wall.

Isotope	Time (min)	
	<i>D. parva</i>	<i>C. submarinum</i>
¹⁴ C-Methylamine	40	300
¹⁴ C-Benzoic Acid	30	360
³ H ₂ O	10	10
³ H-Dextran	10	10
³ H-TPP ⁺	180	240

Table 4.2: Length of Time Required for Optimum Uptake of the Isotopes in *D. parva* and *C. submarinum*. Summary of time courses in Figures 4.2 (a, b).

4.2.3 Effects of unlabelled Dextran, TPP⁺, Methylamine and Benzoic Acid on *D. parva* and *C. submarinum*.

An experiment was set up to ensure that the radioactive markers used in the silicone oil experiments produced no detrimental effects on the algal samples. Non-radioactively labelled TPP⁺, Methylamine, Dextran and Benzoic Acid were added in very high concentrations to algal samples. These samples were then placed in the oxygen electrode and oxygen evolution and uptake were measured. The algal samples were all concentrated to 30 µg Chl ml⁻¹. This level of chlorophyll, and therefore cell

number, was much lower than the levels used in the silicone oil experiments (Table 2.1). The concentrations of the unlabelled compounds in this experiment were considerably higher than those used in the silicone oil experiments (Table 4.3). Initially all unlabelled compounds were concentrated to 990 mM but it was not possible to concentrate the dextran to 990 mM because this compound had a very high molecular weight and thus would not readily dissolve. High concentrations of TPP⁺ and Methyalmine produced a detrimental effect on the respiration of *C. submarinum* therefore the molarities of these compounds were reduced. At these lower molarity levels the concentrations of unlabelled compounds present in this experiment were still many orders of magnitude higher than those used in the silicone oil experiments.

Oxygen evolution and uptake of the algae were measured after predetermined incubation times. These time periods corresponded to the lengths of time that the algae would be exposed to the radiolabelled compounds during a silicone oil experiment (Table 4.2). In samples that stood for a long time period oxygen accumulation reached levels that were too high to be measured in the oxygen electrode. These samples required nitrogen gas to be bubbled through them before oxygen evolution and uptake could be measured. The nitrogen gas displaced the oxygen gas that had accumulated in the sample therefore allowing a measurement to be taken. The results are shown in Table 4.4.

It was clear that the isotopes used in the silicone oil experiments had no detrimental effect on the physiology of the algae, especially at the low concentrations used in the silicone oil experiments.

	<i>D. Parva</i>	<i>C. Submarinum</i>	Molarity in Silicone oil experiments for both species.
Methylamine /ETOH	990 mM	24.8 mM	34.29 nM
Dextran /H ₂ O	32.7 mM	32.7 mM	8.0 μM
Benzoic Acid /H ₂ O	990 mM	990 mM	8.16 nM
TPP ⁺ /ETOH	990 mM	24.8 mM	6.04 nM

Table 4.3: Concentrations of the radiolabelled markers used in the silicone oil experiments (nM or μM) compared to nonlabelled markers used to test their toxicity (mM).

4.2.4 Growth Conditions for Internal pH and Membrane Potential Experiments.

For most of these experiments, algal growth was subjected to two conditions: steady state and shock.

i) Steady state conditions

Algae were grown in pH 5.5, 7.5 and 9.0 media. For pH 5.5 and 9.0 growth took place in a fermenter set up for batch culture growth (sections 2.2.1.2 and 2.2.2.2). The

	<i>D. parva</i>		<i>C. submarinum</i>	
	Photosynthesis	Respiration	Photosynthesis	Respiration
Control + ETOH	31.67 ± 1.26	8.61 ± 0.83	42.08 ± 2.19	11.69 ± 0.48
Dextran	50.96 ± 1.26	12.16 ± 0.62	54.35 ± 0.415	15.20 ± 0.95
Control	44.32	7.84	46.03	15.35
Benzoic Acid	59.53 ± 3.52	14.44 ± 2.35	58.53 ± 2.73	15.19 ± 1.26
Control	53.20	6.84	31.15	8.41
Methylamine	64.86 ± 2.87	19.25 ± 2.17	33.94 ± 1.63	11.53 ± 0.269
Control	26.6	5.32	21.25	7.415
TPP ⁺	26.56 ± 3.44	9.12 ± 0.72	31.63 ± 0.47	5.60 ± 0.71

Table 4.4: The effects of the non Labelled Compounds on both Photosynthesis and Respiration in *D. parva* and *C. submarinum*. Oxygen evolution of two control samples were measured before the first replicate of each new group. This was done to ensure that the standing time taken did not have a detrimental effect on the algal sample. Only two control measurements were taken, therefore no error of these controls were calculated. The isotopes methylamine and TPP⁺ were resuspended in ETOH. The remainder were resuspended in H₂O. The concentrations of non-labelled compounds used are given in Table 4.5.

fermenter ensured the maintenance of these pH levels by the automatic addition of alkali (1 M NaOH) or acid (1 M HCl) when required. The growth of pH 7.5 algal cells took place in flask batch culture.

ii) Shock conditions

Algae were grown in pH 7.5 medium in flask batch culture and for the purposes of this experiment resuspended in growth medium with values of 5.5, 7.5 or 9.0. The differences in pH media were achieved using Tris buffer and the addition of HCl or NaOH after autoclaving. The samples were resuspended in the new pH medium for a minimum of three hours before being used in experiments.

4.2.5 Internal pH

Internal pH of Steady State Cells

Figure 4.3 shows that both *D. parva* and *C. submarinum* cells adapted to pH 7.5 and 9.0 media were able to maintain a near neutral internal pH. Both algae produced very similar results, but *D. parva* was able to maintain a more neutral internal pH than *C. submarinum* at an external pH of 9.0 although the error found was large.

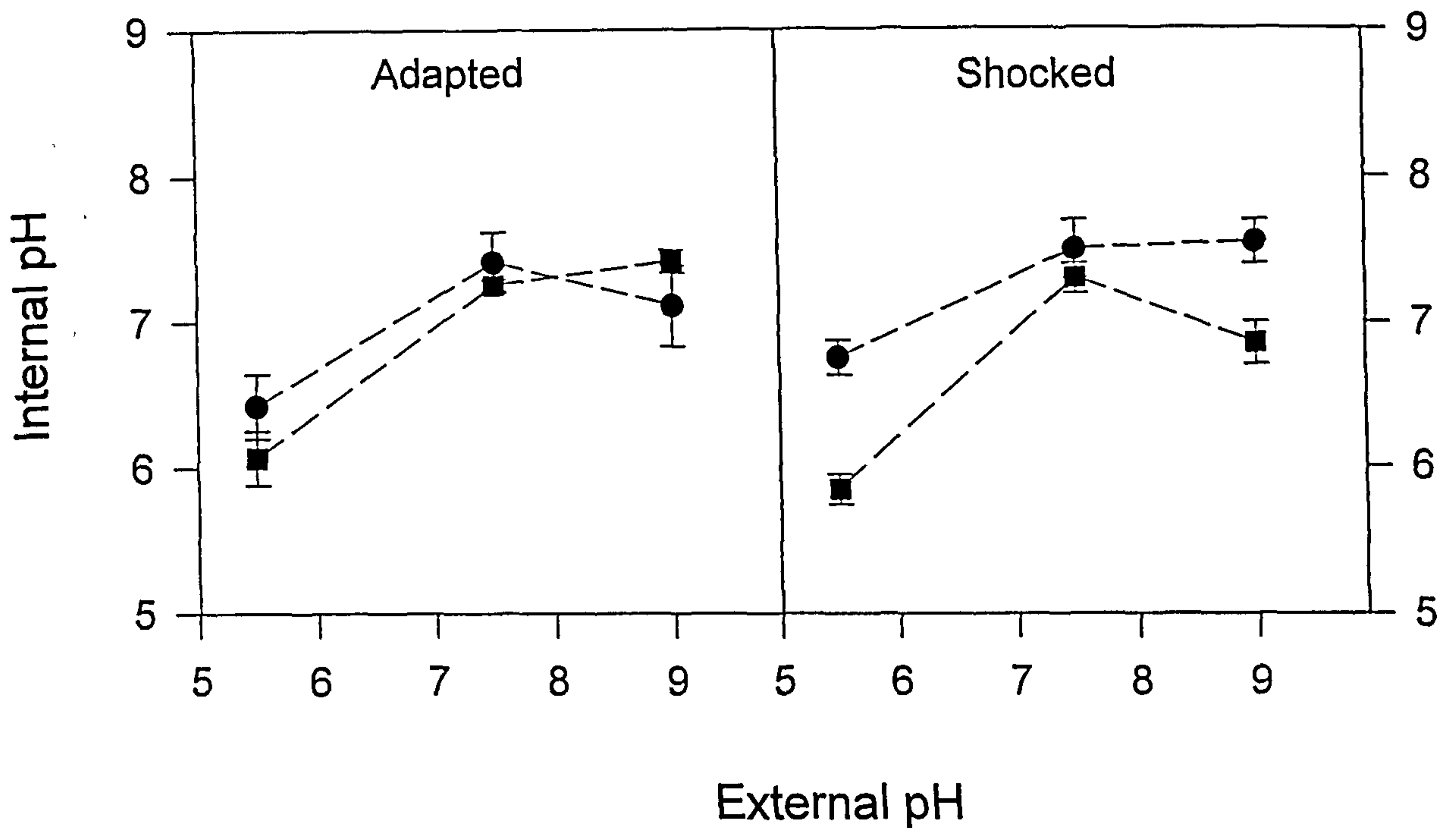


Figure 4.3: Internal pH Measurements for *D. parva* and *C. submarinum* Cells Adapted to or Shocked into pH Media 5.5, 7.5 and 9.0. ● = *D. parva*, ■ = *C. submarinum*. Shocked cells were resuspended in the fresh medium for 3 hours before measurements were taken.

Both algae grown at pH 5.5 showed a significantly reduced internal pH when compared to the internal pH of neutrally grown cells (*D. parva* - $P = 0.016$; *C. submarinum* - $P = 7.52 \times 10^{-5}$). This lower internal pH was about one pH unit higher than the external pH for *D. parva*, but only about 0.5 pH units higher for *C.*

submarinum. This suggests that these algae were better able to maintain a near neutral pH and survive at high external pH levels than low external pH levels (Figure 4.2). This contradicts some of the results found in Chapter 3 where the electron micrographs for *D. parva* at extremes of pH appeared to be stressed, although *C. submarinum* was less visibly stressed (Figures 3.4 and 3.7). The growth curves (Figure 3.1) also showed *D. parva* to have reduced rates of growth at alkaline and acid external pH media. The rates of growth for *C. submarinum* remained unaffected.

Internal pH of Shocked Cells

The internal pH of *D. parva* cells were found to be maintained at a near neutral level when the pH 7.5 grown cells were shocked into pH 5.5 and pH 9.0 media, although there was a small decrease in internal pH at an external pH of 5.5 (Figure 4.3).

In contrast, *C. submarinum* cells showed a large decrease in internal pH at an external pH of 5.5 and a surprising decrease in internal pH at pH 9.0. The error calculated for all shocked cells was small (Figure 4.3).

The internal pH of the cells shocked in pH 5.5 media was established to be lower than that found in the pH 7.5 and pH 9.0 shocked cells for both *D. parva* and *C. submarinum*. There was a clear difference between the external pH and the internal pH for the shocked cells indicating that the time period of three hours was long enough for the pH shock to have an effect on the cells.

Internal pH was found to be maintained at a near neutral level for both *Dunaliella parva* and *Chlorococcum submarinum* cells at steady state and under shock conditions for pH 7.5 and 9.0. This maintenance of a neutral internal pH enables the cells to function optimally over a wide pH range. The pH 5.5 adapted cells show reduced levels of internal pH as did the pH 5.5 shocked *C. submarinum* cells. In the case of *C. submarinum*, a pH 5.5 shock had a substantial effect.

4.2.6 Membrane Potential

In all cases the membrane potential was inside negative.

Steady State

Membrane potential for both *D. parva* and *C. submarinum* cells adapted to pH 5.5, 7.5 and 9.0 was seen to increase as the external pH increased (Figure 4.4). *C. submarinum* showed a larger membrane potential than *D. parva* for all external pH media. This difference was greatest with growth media of pH 9.0.

Shock

D. parva and *C. submarinum* showed similar responses in decreasing their membrane potential when the external pH was changed from 7.5 to 5.5. The membrane potential of *C. submarinum* was larger than that of *D. parva* at both pH 5.5 and 7.5 (Figure 4.4).

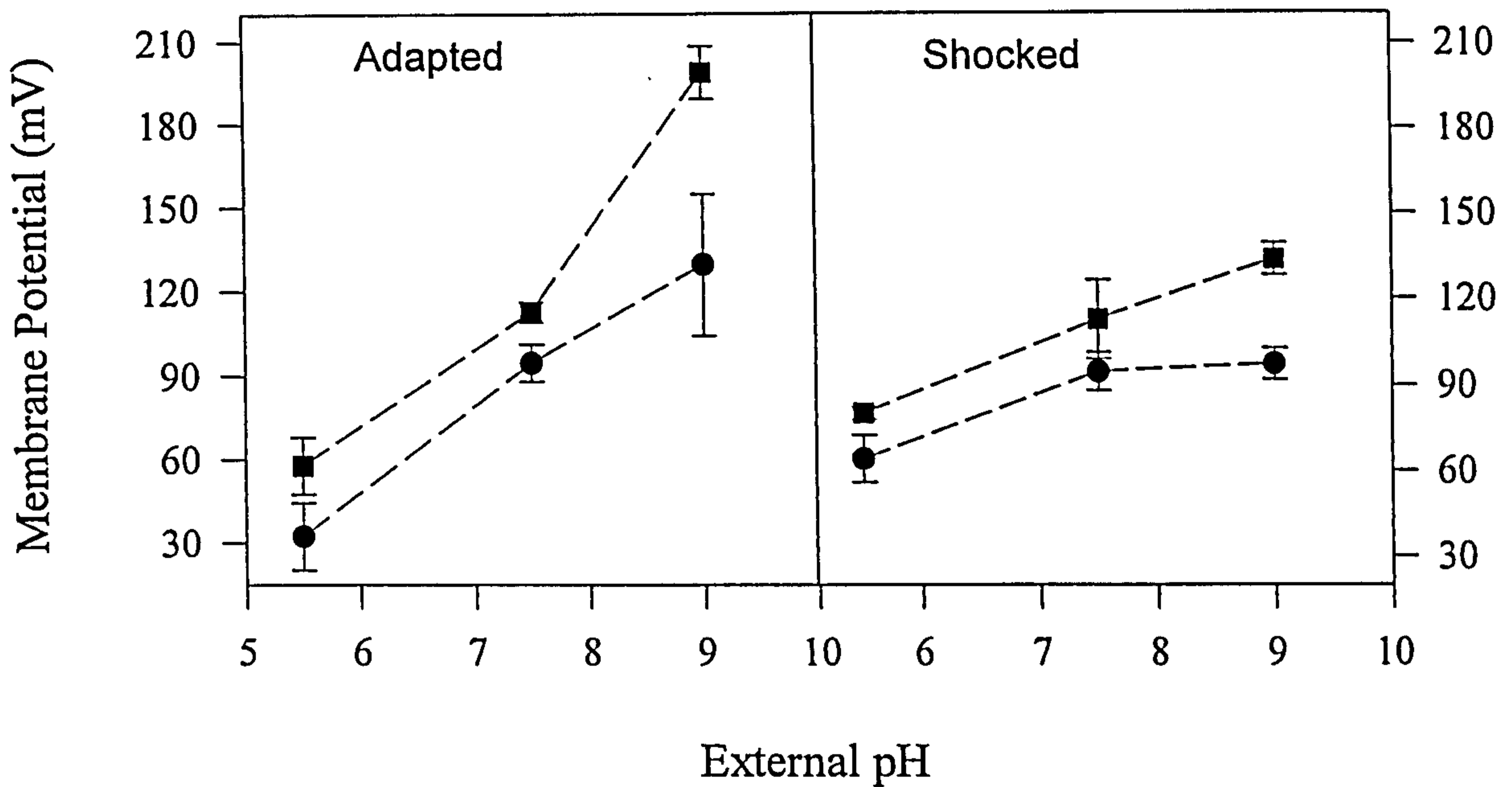


Figure 4.4: Membrane Potential Measurements for *D. parva* and *C. submarinum* Cells Adapted to or Shocked into pH 5.5, 7.5 and 9.0. ● = *D. parva*, ■ = *C. submarinum*. Shocked cells were resuspended in the fresh medium for 3 hours before measurements were taken.

The membrane potential of pH 7.5 grown *C. submarinum* cells that were shocked into pH 9.0 medium increased. This large increase was found not to be significant at a 5% confidence level ($P = 0.265$). In contrast, *D. parva* cells did not show this increase in membrane potential for pH 9.0 shocked cells (Figure 4.4) and were found to be statistically similar to the membrane potential of pH 7.5-grown cells ($P = 0.768$).

At steady state and under shock conditions membrane potential for *D. parva* and *C. submarinum* cells was found to increase with an increase in external pH (except for pH shocked *D. parva* cells). The increase in membrane potential is related to the maintenance of a near neutral internal pH of the cell and therefore enables the cell to function as normally as possible.

4.2.7 Intact Chloroplasts

D. parva and *C. submarinum* cells were grown at pH 7.5 and harvested for intact chloroplast preparation (section 2.12) after about seven days growth in flask batch culture. Intact chloroplasts were prepared from pH 5.5 grown *D. parva* cells after about three weeks growth in the fermenter. It was found that if the cells were allowed to grow for two to three weeks a slime surrounded the cells and cell clumping would occur resulting in a very poor chloroplast band or no chloroplast band produced at the Percoll gradient stage. This has been noted also by Goyal *et al.* (1988).

The internal pH and membrane potential of intact chloroplasts of algal cells were measured with the same isotopes used for the whole cells. These measurements were only obtained for *D. parva* grown at pH 5.5 and 7.5, and *C. submarinum* pH 7.5. Only one replicate was measured for *D. parva* pH 5.5. Due to the difficulty in preparing large amounts of intact chloroplasts, no checks could be made on the suitability of the isotopes to measure the membrane potential and internal pH values.

4.2.7.1 Verification of intact Chloroplasts

Microscope

Under the microscope at x 1000 magnification with the aid of oil, it was possible to see the intact chloroplasts. They appeared round in shape with a darker band around the outer edge.

NaH¹⁴CO₃ Fixation

The chloroplasts took up ¹⁴C labelled sodium bicarbonate and therefore must have been intact and able to photosynthesise. The addition of TCA to the intact chloroplasts preparation removed any remaining NaH¹⁴CO₃ as CO₂ gas. This ensured that the only ¹⁴C present after TCA treatment must have been taken up by the intact chloroplasts and fixed into acid stable compounds.

4.2.7.2 Internal pH

The internal pH values of the intact chloroplasts for both algae grown in pH 7.5 media were found to be near neutral (Table 4.5). Both algae grown in pH 7.5 media produced chloroplast internal pH measurements slightly lower than the external pH of the media. These internal pH values were found to be not significantly different to those established for whole cell internal pH values (*D. parva*, P = 0.485; *C. submarinum*, P = 0.397).

	<i>D. parva</i> pH 7.5	<i>D. parva</i> pH 5.5	<i>C. submarinum</i> pH 7.5
Membrane Potential	-80.20 ± 1.64 (-94.39 ± 6.60)	-87.44 (-32.42)	-71.65 ± 5.98 (-112.49 ± 3.86)
Internal pH	7.34 ± 0.04 (7.41 ± 0.21)	4.20 (6.42)	7.02 ± 0.37 (7.25 ± 0.04)

Table 4.5: Membrane Potential and Internal pH for Intact Chloroplasts of *D. parva* Cells Grown at pH 5.5 and 7.5 and *C. submarinum* Cells grown in pH 7.5 medium. The numbers in brackets are the membrane potentials and internal pH values for intact cells grown at the same external pH values.

The internal pH of chloroplasts isolated from pH 5.5 grown *D. parva* cells was found to be lower than that found for whole cells. To assume that this measurement is accurate many more replicates would have to be taken.

4.2.7.3 Membrane Potential

The membrane potential of the intact chloroplasts isolated from *D. parva* and *C. submarinum* cells grown in pH 7.5 medium were found to be very similar. When compared to the membrane potential of whole cells the membrane potentials for *D. parva* chloroplasts were found to be similar ($P = 0.908$) but those for *C. submarinum* chloroplasts were found to be significantly lower ($P = 0.002$).

The membrane potential of the intact chloroplasts from *D. parva* pH 5.5 grown cells was much higher than that found for intact cells but very similar to the membrane potential of the intact chloroplasts isolated from pH 7.5 grown cells and whole pH 7.5 grown cells (Table 4.5). This may suggest that the pH of the cytoplasm is at a neutral pH level. Many more replicates would have to be measured to assume that this measurement is accurate.

The results produced for the intact chloroplasts were of great interest and it is unfortunate that the cell material required was not available to complete the data set. Further studies in this area could produce internal pH and membrane potential results for cells grown in different pH media. These experiments were not possible in this study because the amount of material required for each experiment, and the length of time required to grow the cells made this an unfeasible proposition. Also if the algae were grown in the fermenter for two to three weeks before the samples were removed, it was found that the cells were surrounded by slime making it almost impossible to produce a clear band when centrifuged through the Percoll gradient.

The techniques involved in the isolation of chloroplasts produced intact chloroplasts, but at each step in the protocol chloroplasts were damaged or destroyed. Improvements in technique would increase the number of viable intact chloroplasts and allow further experimental studies to be undertaken. Repeated pelleting damaged the chloroplast envelope (Price and Reardon, 1982) and therefore maybe it could be possible to remove one, or more, of the pelleting stages. After the cells had been broken the brakes on the centrifuge were switched off to help minimise pelleting damage. Maybe also the use of swinging bucket rotors would help minimise damage to the chloroplasts.

4.3 Conclusions

The use of radiolabelled isotopes in the silicone oil experiments described in the present study produced plausible results. The internal pH for *D. parva* and *C. submarinum* cells studied was closer to a neutral pH than the external pH value suggesting that the cells actively tried to maintain a near neutral internal pH (Figure 4.3). The maintenance of a near neutral internal pH may be the result of the cell membrane being largely impermeable to H^+ or the algae may be capable of pumping H^+ in or out of the cells. A passive H^+ -influx into the cell was thought to increase with increasing external H^+ concentration (i.e. more acid external pH) and in order to maintain a constant cytoplasmic pH the cells would be required to re-export increasing amounts of H^+ per unit of time and unit of surface area with decreasing external pH

(Gimmler *et al.*, 1988). An ATP driven pump is thought to be responsible for the pumping out of excess H^+ (Nicholls and Ferguson, 1992).

The internal pH measurements from *D. parva* for both steady state and shocked cells were more neutral than those for *C. submarinum* (Figure 4.3). This indicates that either *D. parva* is better able to maintain a near neutral internal pH or that *C. submarinum* is better able to function over a range of internal pH values. *C. submarinum* has been found by Blackwell and Gilmour (1991a) to be able to survive and grow in a wide pH media range (pH 2-10.5), unlike *D. parva* which was unable to survive at pH values below 3 (Gimmler *et al.*, 1990). The reason for cell death was concluded to be internal acidification and accumulation of toxic cations.

It would have been ideal to measure the internal pH of the cells with a second method either by another measurement of ion distribution or by the use of an internal pH indicator (Rottenberg, 1979). Weiss and Pick (1991) used a fluorescent indicator (atebrin) to measure the internal pH of *D. salina* cells at alkaline external pH. This dye accumulated in the acidic vacuoles and did not enter the chloroplast. Gimmler *et al.* (1988) obtained cytoplasmic pH values of pH 7.0 for *D. parva* in the presence of a wide range of external H^+ concentrations using *in vivo* ^{31}P -NMR spectroscopy and the DMO method.

The membrane potentials of the cells at steady state and those shocked into a range of external pH media showed a similar pattern (Figure 4.4). At high external pH the membrane potential of the cells was found to be highest and at low external pH the

membrane potential was lowest. The membrane potential of the shocked cells did not cover the same range as the membrane potentials of the steady state cells. This indicates that although these shocked cells had been exposed to their new medium for three hours the cells had not yet become fully adapted to the new external pH. If these shocked cells had been resuspended in their new medium for longer the membrane potentials of these samples may have been more similar to the steady state cells. The membrane potentials of the *C. submarinum* cells were higher than the *D. parva* cells over the entire pH range this may be responsible for the lower internal pH values found for *C. submarinum*. It would have been useful to study a second method, if possible, for measuring membrane potentials to back up these results.

The membrane potential is thought to be pH dependent in algae being larger at more alkaline values of external pH. This is also true in bacteria, but it has been suggested that in extremely alkalophilic bacteria membrane potentials do not offset increasingly adverse differences in pH (Kroll, 1990; Grant and Horikoshi, 1992).

Dunaliella acidophila, an extremely acid resistant strain of *Dunaliella*, that exhibits optimal growth and photosynthetic production at pH 1, possesses a positive membrane potential in the range of 50 to 100 mV (Remis *et al.*, 1994). *D. acidophila* has several adaptations to maintain a large trans-membrane H^+ gradient and allow the cells to survive. H^+ influx into the cell must be minimised by a low permeability coefficient of the plasma membrane for H^+ . Protons that have entered the cell must be pumped out, a process which requires energy (Gimmler *et al.*, 1989). Sekler *et al.* (1991) suggested that in *D. acidophila* the H^+ pump is likely to be the major, and possibly only,

mechanism to sustain the pH gradient of 6-7 pH units as the optimum external pH is 1 and the internal pH is maintained at pH 7.0.

Effect of changing pH levels on
intracellular enzymes extracted
from *Dunaliella parva* and
Chlorococcum submarinum.

CHAPTER FIVE

5.1 Introduction

It has been shown in the previous chapters that the internal pH of the algal cells remains at near neutral pH even when the external pH of the medium is alkaline. When in an acidic medium the internal pH of the cells was found to be at a higher pH level than the external medium although at a lower pH level than neutral. To further check these results, enzyme activities in crude extracts were measured to establish the potential effects of external pH changes on metabolic pathways. The activities of these enzymes, taken from different organelles in the cell, could also help determine the uniformity of internal pH within the different compartments of the cell.

Five enzymes have been studied:

<u>Enzyme</u>	<u>Cycle</u>	<u>Organelle</u>
Malate dehydrogenase	Citric Acid Cycle	Mitochondria
Fumarase	Citric Acid Cycle	Mitochondria
Glycerol P-dehydrogenase	Glycerol cycle: 1st half of cycle	Chloroplast
Dihydroxyacetone (DHA) reductase	Glycerol cycle: 2nd half of cycle	Cytoplasm
Acid phosphatase	General purpose	Throughout cell

This approach has been used before by Gimmler *et al.* (1983) who studied the effect of different external salt concentrations on the intracellular enzymes of *D. parva*.

5.2 Results and Discussion

The results were calculated in $\mu\text{mol}^{-1} \text{min}^{-1}$ for 1×10^6 cells, using the equation given in section 2.13.2. For all enzyme activity experiments algal growth was subjected to two conditions.

Steady state conditions

Algae were grown in media with pH values of 5.5, 7.5 and 9.0. For media pH 5.5 and 9.0 the growth took place in a fermenter set up for batch culture growth (sections 2.2.1.2 and 2.2.2.2). The fermenter ensured the maintenance of extreme pH levels by automatic addition of acid (1 M HCl) or alkali (1 M NaOH) when required. pH 7.5 growth took place in 250 ml flasks (sections 2.2.1.1 and 2.2.2.1).

Shock conditions

Algae were grown in pH 7.5 medium in flask batch culture and for the purposes of this experiment resuspended in growth media set at pH 5.5, 7.5 or 9.0. The samples were resuspended in the new pH medium for a minimum of three hours before activity was measured.

5.2.1 Malate dehydrogenase

This enzyme catalyses the oxidation of malate to oxaloacetate in the Citric Acid cycle but because the equilibrium of the reaction lies far to the left (malate formation), the reaction is usually studied by measuring the oxidation of NADH in the presence of oxaloacetate (Reeves *et al.*, 1971).

D.parva

The activity of the enzyme malate dehydrogenase in *D. parva* samples for both steady state cells and shocked cells produced a similar pattern (Figure 5.1). The highest levels of activity were found in pH 5.5 shocked and adapted cells and the lowest rates were recorded in pH 9.0 shocked and adapted cells. The error found in the steady state cells was large suggesting a large intersample variation. The malate dehydrogenase activity of pH 9.0 and pH 7.5 adapted cells was established to be not quite significantly different at 5% ($P = 0.0598$).

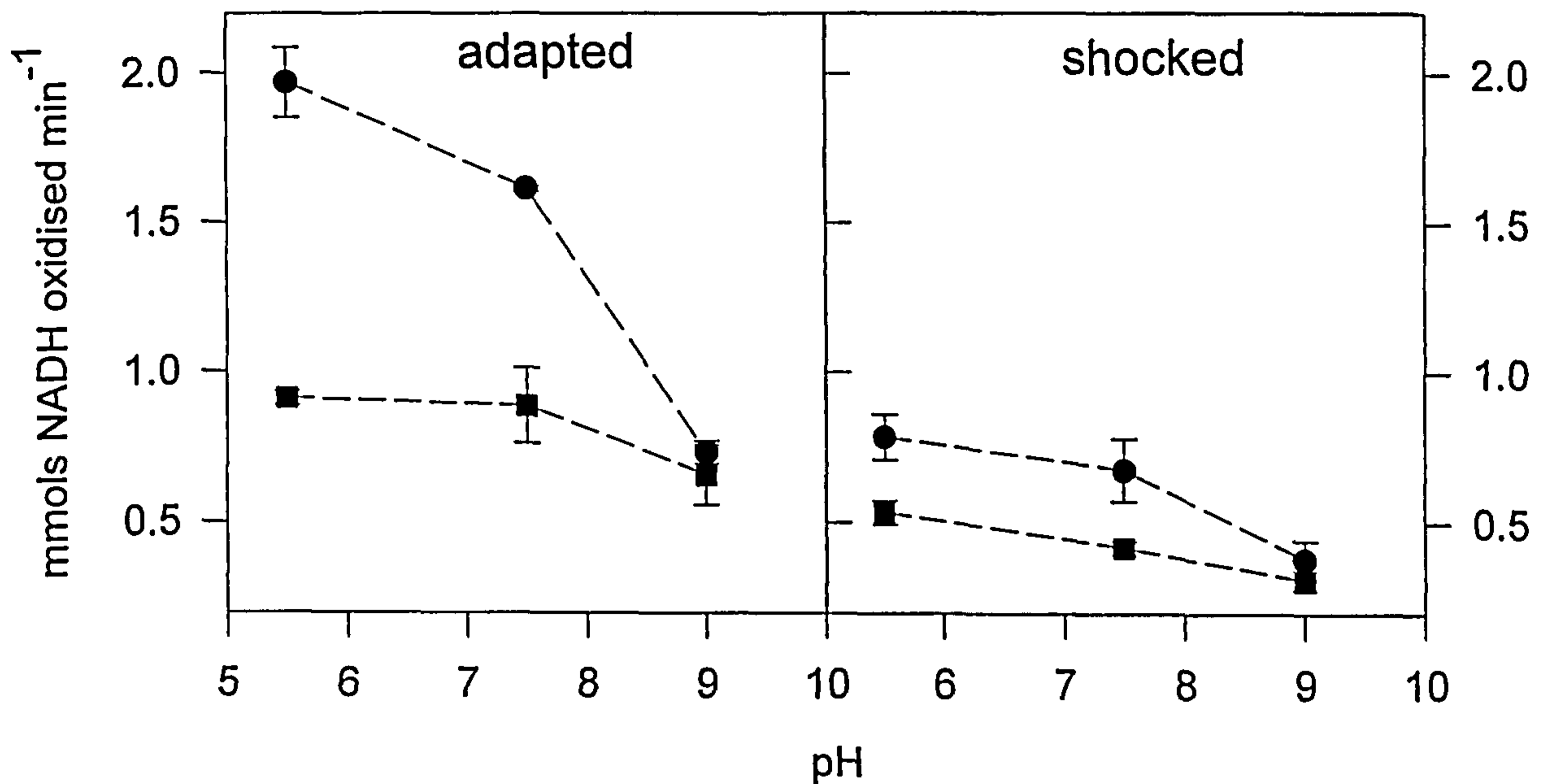


Figure 5.1: Malate dehydrogenase activity in *D. parva* and *C. submarinum* in steady state and shock conditions. *D. parva* = ●, *C. submarinum* = ■. All activities are for 10^6 cells.

C. submarinum

The activity of malate dehydrogenase in *C. submarinum* adapted cells produced a similar pattern to that of *D. parva*; highest levels of activity were found at pH 5.5 and the lowest levels found at pH 9.0. The highest level of activity in *C. submarinum* shocked cells were found in those resuspended in pH 5.5 medium and the lowest in pH

9.0 resuspended cells. The level of activity in the *C. submarinum* shocked cells was lower than that found in *D. parva* shocked cells (Figure 5.1). There was very little difference in the malate dehydrogenase activity for cells resuspended in pH 5.5 or 7.5 ($P = 0.762$).

Both algae produced similar patterns, although the activity levels measured in *C. submarinum* were found to be lower than the activity in *D. parva* cells at pH 5.5 and 7.5 (Figure 5.1). In pH 9.0 adapted cells malate dehydrogenase activity in *D. parva* and *C. submarinum* was found to be not significantly different at 5% level ($P = 0.413$).

5.2.2 Fumarase

Fumarase is found in the matrix of mitochondria (Stitt, 1984), and it catalyses a hydration reaction in the Citric Acid cycle. This is the reaction immediately prior to the conversion of L-malate to oxaloacetate (section 5.2.1).

D. parva

Fumarase activity in *D. parva* adapted cells increased significantly with an increase in external pH from pH 7.5 to 9.0 ($P = 0.006$), and there was a non-significant increase from pH 5.5 to 7.5 medium ($P = 0.158$) (Figure 5.2). In *D. parva* shocked cells (Figure 5.2), the activity was found to decrease with the increase in external pH. The error in all of the shocked samples was small and the most significant difference between samples occurred between the pH 7.5 and pH 9.0 shocked ($P = 0.0566$).

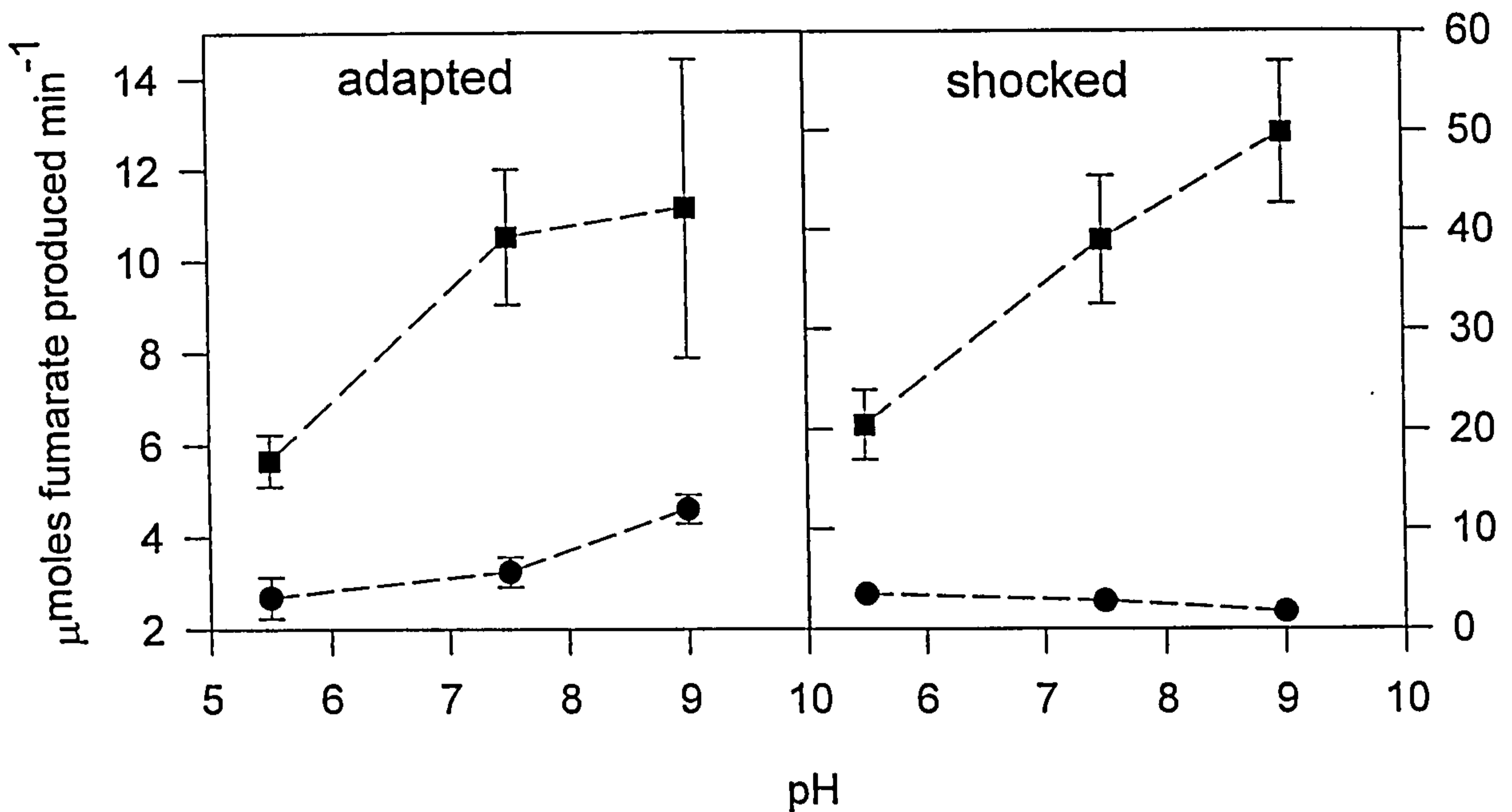


Figure 5.2: Fumarase activity in *D. parva* and *C. submarinum* in steady state and shock conditions. *D. parva* = ●, *C. submarinum* = ■. All activities are for 10^6 cells.

C. submarinum

The highest levels of activity of fumarase in both shocked and adapted *C. submarinum* cells was at pH 9.0. The lowest rate of activity was found in pH 5.5 cells shocked and adapted cells. This was the same as the pattern produced for adapted *D. parva* cells (Figure 5.2). The level of activity in all of the *C. submarinum* cells was much higher than that found in *D. parva* cells.

Errors could have arisen due to sedimentation of protein during the assay (Stitt, 1984), thus the activity of the enzyme was measured as soon as the assay had settled. The fumarase assay was very sensitive to temperature, reagents and cuvettes (Stitt, 1984). In these experiments the same quartz cuvette was used throughout, as were the same reagents. Therefore the temperature was not controlled and this could have lead to errors occurring.

5.2.3 Dihydroxyacetone (DHA) reductase (also called Glycerol Dehydrogenase)

Dunaliella species are able to survive extreme salt stress by keeping their internal NaCl level much lower than that of the external medium and by synthesising large amounts of glycerol for osmotic adjustment (section 1.3) (Gimmler *et al.*, 1983; Gilmour, 1990). Glycerol is removed from the cell by being converted to starch. The start of this process is catalysed by the NADP-specific DHA reductase (Brown *et al.*, 1982). This enzyme is found exclusively in the cytoplasm of the cell. Results were obtained for *D. parva* for both adapted and shocked cells (Figure 5.3). Using the same method no activity of this enzyme was detected for *Chlorococcum submarinum* grown in 0.5 M NaCl media. It is known that *C. submarinum* produces glycerol in response to salinity increase (Blackwell and Gilmour, 1991b) but it has not been proven that production is through this cycle. Using a more sensitive method (AMINCO DW 2000) DHA reductase activity in *C. submarinum* was found (0.191 μ moles NADPH

oxidised min^{-1} for 1×10^6 cells). These rates were much lower than those found in *D. parva* ($0.983 \mu\text{moles NADPH oxidised min}^{-1}$ for 1×10^6 cells).

D. parva

DHA reductase activity in adapted cells was found to be very similar for all three pH growth levels (Figure 5.3). pH 9.0 adapted cells produced the greatest activity and pH 5.5 adapted cells the lowest, although these results were not significantly different at 5% level ($P = 0.385$). These data support the results of the glycerol measurements on cells grown at different pH levels (section 3.2.9). The highest rates of activity of DHA reductase, which indicates a high turnover of the glycerol cycle, was found in pH 9.0 *D. parva* cells. The highest level of glycerol found in *D. parva* cells was also in pH 9.0 cells. This suggests that an error may not have occurred in the glycerol measurements, as previously thought (section 3.2.9).

In the shocked *D. parva* cells DHA reductase activity was greatest in pH 7.5 cells and lowest in pH 9.0 cells.

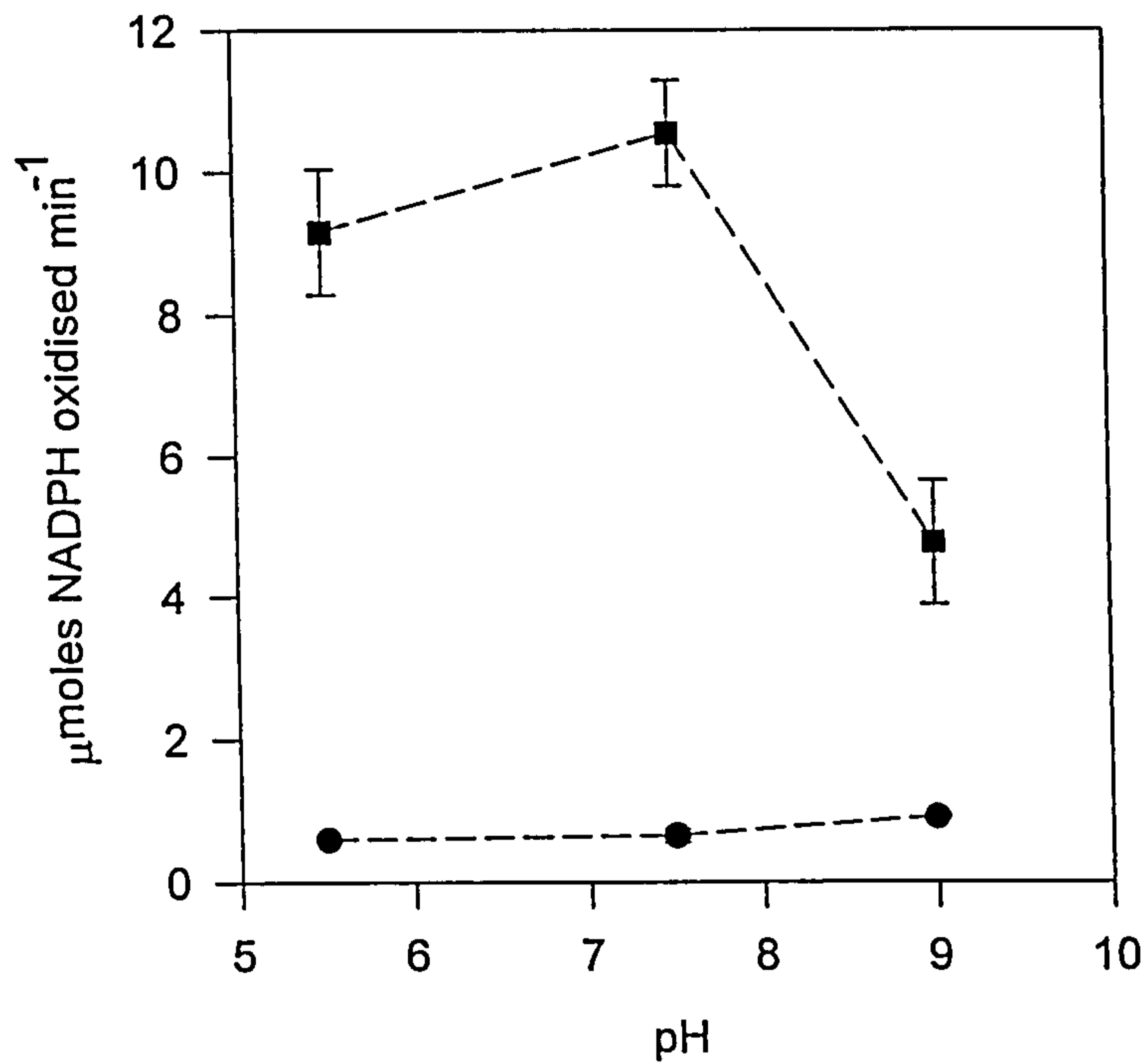


Figure 5.3: DHA reductase activity in *D. parva* in steady state and shock conditions. Adapted cells = ●, Shocked cells = ■. All activities are for 10^6 cells.

5.2.4 Glycerol Phosphate-dehydrogenase

D. parva

Glycerol P-dehydrogenase is located in the *Dunaliella* chloroplast (section 1.3) (Ginzburg, 1987). This enzyme catalyses a reversible reaction on the pathway leading

to glycerol synthesis and converts dihydroxyacetone phosphate to glycerol phosphate, with the help of NADH (Ginzburg, 1987; Avron, 1992).

This enzyme was only very briefly studied. It was established that the enzyme was present and active in *D. parva* cells grown in pH 7.5 media: 6.46 $\mu\text{mols NADH oxidised min}^{-1} 10^6$ cells. No activity was found in *C. submarinum*.

5.2.5 Flagellar ATPase

D. parva

The intention had been to measure the ATPase activity associated with the flagella of *D. parva*, and study the effects of external pH on this activity. Unfortunately, measurements were not possible because when the algal sample was removed from the fermenter *D. parva* was found to be non-motile. The agitator in the fermenter provided constant movement for the alga and this apparently inhibited their use of flagella, alternatively the continuous movement of the agitator in the fermenter damaged or destroyed the flagella. Flask batch culture was not used as the media were unable to be maintained at the required external pH (Section 2.2.3).

5.2.6 Acid Phosphatase

D. parva

Acid phosphatase activity in *D. parva* cells, adapted to their external pH, was found to increase as the external pH increased (Figure 5.4). The activities measured for pH 7.5 and 9.0 grown cells were found not to be significantly different at the 5% level ($P = 0.498$). For pH shocked *D. parva* cells the activity was found to peak at pH 7.5 and be lowest at pH 5.5 (Figure 5.4).

C. submarinum

C. submarinum activity for both pH adapted and shocked cells was found to peak at pH 7.5 (Figure 5.4). In the adapted cells the lowest activity rates recorded were for pH 5.5 cells. The pH shocked cells showed the lowest activity in the pH 9.0 cells although the activity for these cells was not significantly different to that found for pH 5.5 shocked cells ($P = 0.610$).

It can be seen from the data for adapted cells that *C. submarinum* produced higher levels of activity than *D. parva* cells. However, in shocked cells *D. parva* produced higher activity levels than *C. submarinum* (Figure 5.4).

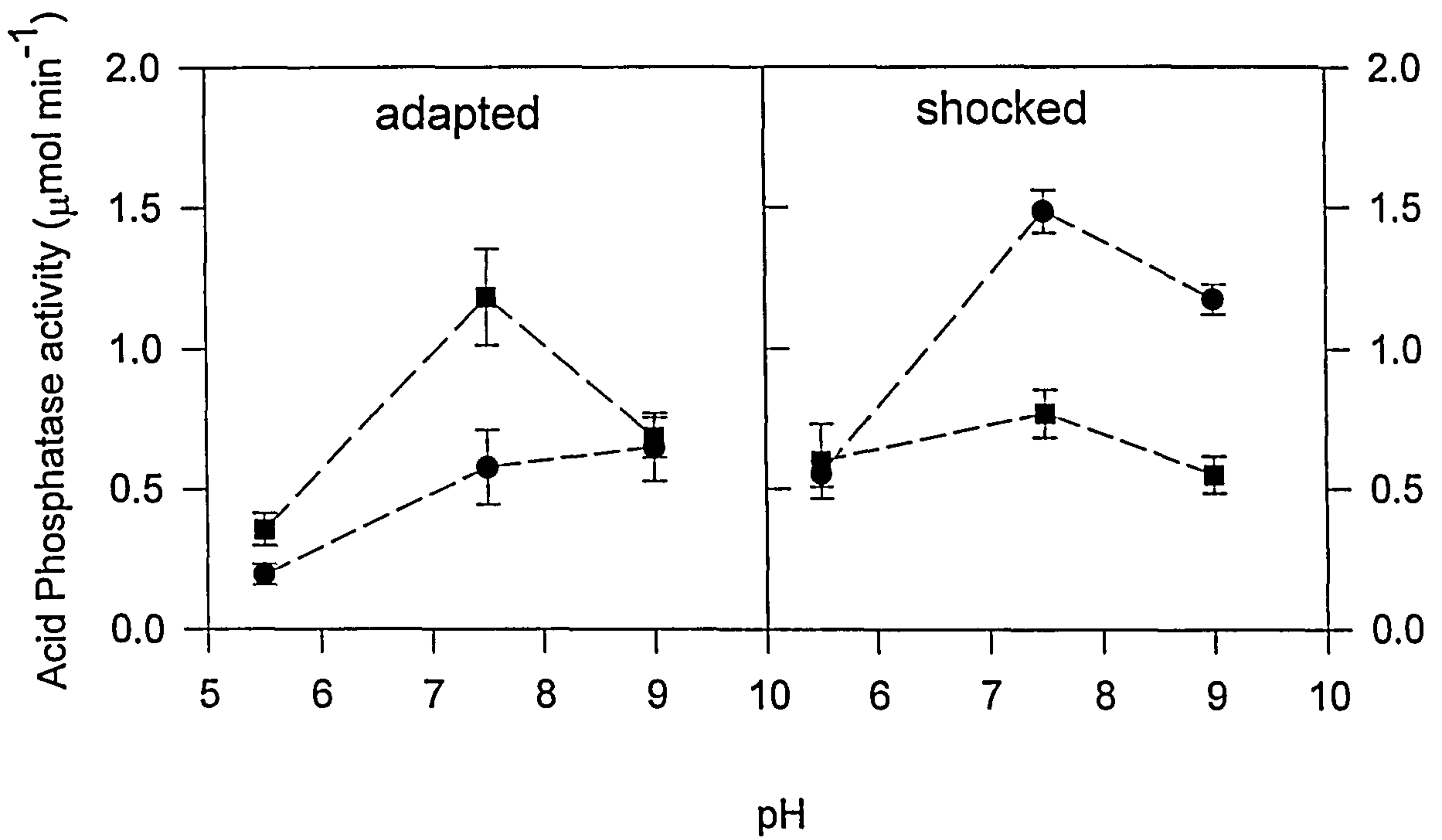


Figure 5.4: Acid phosphatase activity in *D. parva* and *C. submarinum* in steady state and shock conditions. *D. parva* = ●, *C. submarinum* = ■. All activities are for 10^6 cells.

5.3 Conclusions

The study of enzyme activity in several cell compartments should help establish the optimum pH for enzyme activity in each of the organelles studied, and determine whether the established internal pH values are uniform throughout the cell.

Table 5.1 shows the optimum pH values for each of the enzymes studied for both algae in shock and adapted conditions. The intracellular enzymes in these algae differ in their response to external pH although both algae largely produce the same optima for the same enzyme. In shock experiments, pH 7.5 cells should produce the highest levels of activity because resuspension in pH 7.5 medium is the least damaging. When the optimum is not pH 7.5 then this is a direct effect of external pH.

Malate dehydrogenase was found to be more resistant to acid pH values (Figure 5.1). It showed an optimum at pH 5.5 in both adapted and shock conditions for both algae. Fumarase was found to be more resistant to higher pH, with one exception, *D. parva* shock conditions (Figure 5.2). This is interesting because both are found in the mitochondria and are involved in the Citric Acid cycle, the malate dehydrogenase reaction following that of fumarase.

The highest level of DHA reductase activity was found in pH 7.5 shocked cells (Figure 5.3). This was no surprise as the cells were grown in pH 7.5 medium and pH shock would not be expected to stimulate the production of glycerol. DHA reductase

Enzyme	Optimum pH			
	<i>D. parva</i>		<i>C. submarinum</i>	
	Adapted	Shocked	Adapted	Shocked
Malate Dehydrogenase	5.5	5.5	5.5	5.5
Fumarase	9.0	5.5	9.0	9.0
DHA Reductase	9.0	7.5	-	-
Acid Phosphatase	9.0	7.5	7.5	7.5

Table 5.1: Summary of optimum enzyme activities in *D. parva* and *C. submarinum*.

adapted cells produced surprising results, with the optimum being pH 9.0. This data supports the glycerol production measurements in Chapter 3 (section 3.2.9) where the highest levels of glycerol found were in the pH 9.0 grown cells.

Acid phosphatase, a general purpose enzyme, functioned best at neutral pH for both algae in adapted and shock conditions (Figure 5.4). The only exception to this was adapted *D. parva* cells which showed an optimum of pH 9.0, but this was found not to be significantly different at the 5% level to pH 7.5 grown cells ($P = 0.498$).

It must also be noted that the two algae were grown in media with different salt concentrations and this may have affected the activity of the intracellular enzymes. This would certainly be the case for Glycerol P-dehydrogenase which is involved in the production of glycerol which is greater at higher external salt concentrations. It may also be true for DHA reductase, which is involved in the removal of glycerol, since the turnover of glycerol is likely to be quicker at high salinities. The enzymes glycerol P-dehydrogenase and DHA reductase were studied in *C. submarinum* grown in 0.5 M NaCl medium instead of 0.25 M NaCl and still there was no measurable activity using the Ultraspec II LKB spectrophotometer. It was attempted to grow *C. submarinum* at even higher salt concentrations but growth was very slow if non-existent. This contradicts the findings by Blackwell and Gilmour (1991a), which indicated that *C. submarinum* could grow well at 1.0 M NaCl.

DHA reductase and glycerol P-dehydrogenase from the glycerol cycle may be present in *C. submarinum* but at such low levels that was impossible to measure their activity. Perhaps *Chlorococcum* does not produce glycerol by the existing glycerol cycle, but has its own unique glycerol cycle. If glycerol is produced by the original cycle, then the enzymes involved in the production of glycerol should be active in measurable amounts. The enzymes studied were found to be present in *D. parva* (as expected) and therefore they too would be expected at measurable levels in *C. submarinum*. Some activity was found in *C. submarinum* using the AMINCO DW 2000 spectrophotometer but at very low levels. The levels were too low to positively identify that the enzymes were present. These results raise the possibility that a

different pathway of glycerol production or degradation exists in *Chlorococcum*. Another possibility is that the enzymes became inactivated during cell breakage for *C. submarinum*, (Mouget *et al.*, 1993) but this was unlikely because activity was measured for other intracellular enzymes in *C. submarinum* using this method and some of these activities were found to be greater than those found in *D. parva*.

It can be seen from the data in this chapter that the enzyme activity in the cells resuspended in different pH medium (shocked) was found to be much greater than the activity found in the adapted cells measured for most of the enzymes examined.

Malate dehydrogenase activity in pH 7.5 *D. parva* and *C. submarinum* cells resuspended in fresh medium was double that of the pH 7.5 grown cells (Figure 5.1). The DHA reductase activity in the shocked *D. parva* cells was up to ten times greater than the activity found in the pH adapted cells (Figure 5.3). *C. submarinum* showed elevated acid phosphatase levels in the shocked condition as did *D. parva*, but the increase in activity in the *D. parva* shocked cells was found to be much greater (Figure 5.4). Fumarase activity in *D. parva* did not produce the same increase in activity in shocked cells in contrast to the other enzymes studied (Figure 5.2) unlike the fumarase activity in *C. submarinum* shocked cells was found to be at an elevated level.

This increase in activity may be a result of several factors singly or in combination. All of the cells were resuspended in fresh medium. The adapted cells in medium of the same pH and the shocked cells in a medium of a different pH. The resuspension in fresh medium may have encouraged growth in the cells and thus increased intracellular

activity. The fresh medium resuspension may also have removed any inhibitors that had accumulated in the medium due to the breakdown of dead cells. The removal of these inhibitors may encourage growth of the cells and ultimately increase enzymatic reactions. The activity in adapted cells was measured immediately after resuspension in the fresh medium unlike the shocked cells which were allowed to stand in the medium for three hours before the measurements were taken. In retrospect the adapted cells should have been allowed to stand in their fresh medium for three hours too. Therefore no direct comparisons could be drawn between the adapted and shocked cells, only trends, could be compared.

The largest differences between the adapted and shocked cells occurred with *D. parva*. This was probably due to the thick cell wall which surrounds *C. submarinum* cells and therefore the cells did not respond so dramatically as it took longer for the changes in external medium to affect the internal mechanisms of the cell.

The evidence from this chapter for the enzymes malate dehydrogenase and fumarase, suggests that using this approach to estimate the pH levels inside organelle is not a reliable one. Although, both of these enzymes are found in the mitochondrion, they had very different pH optima (Table 5.1).

General Discussion

CHAPTER SIX

This study of *Dunaliella parva* and *Chlorococcum submarinum* was initiated to explore the effects of changes in external pH on each of the algae and to compare these effects. The prime difference in cell morphology between these two algae is that *C. submarinum* possess a thick cell wall and *D. parva* does not. It is evident from the results obtained that this thick cell wall helps to protect the intracellular functions of *C. submarinum* cells from the changes in extracellular pH.

The thick cell wall of *C. submarinum* was clearly visible in the electron micrographs (Figures 3.5, 3.6, 3.7). The electron micrographs also showed that at extremes of external pH the contents of *C. submarinum* cells appeared to be largely unaffected by the external pH. In contrast, the electron micrographs of *D. parva* cells, at acidic or alkaline external pH, showed the cell contents to be very different to the pH 7.5 adapted cells. The cells at extreme pH values appeared to be distorted in shape, especially at pH 9.0. The intracellular structures also appeared to be disordered.

Growing of *D. parva* cells in media with a range of pH values (pH 5.5 to 9.0) showed that growth became reduced at the extreme pH values unlike *C. submarinum* cells which showed no decrease in growth when grown in different pH media (Figure 3.1). These data support the proposal that the thick cell wall of *C. submarinum* protects the cells and allows the cells to continue to grow unaffected by changes in the growth media.

The photosynthetic and respiration rates found for *C. submarinum* contradicted the growth curve results and the proposition that the thick cell wall protected the *C. submarinum* cells. The photosynthetic and respiration rates were found to be reduced in adapted cells at extremes of external pH for both algae (Figure 3.8). The reduction was found to be greater in *D. parva* cells suggesting that the thick cell wall may possibly offer some protection to *C. submarinum* cells. The photosynthetic and respiration rates in pH shocked cells were also found to be reduced at extremes of pH shock, but it was also noted that in *C. submarinum* cells there was no immediate reduction in contrast to *D. parva* cells which reacted immediately (Table 3.3). The photosynthesis and respiration rates in pH shocked *C. submarinum* cells were found to become reduced only after three hours resuspension in the fresh medium.

The use of the inhibitors, CCCP and monensin, demonstrated the protective qualities of the thick cell wall of *C. submarinum*. Both algae showed higher resistance to monensin than CCCP. *C. submarinum* cells showed higher resistance to both of these inhibitors when compared to *D. parva* cells (Figures 3.5, 3.6, 3.7). These results indicated that CCCP and monensin were more readily excluded from the *C. submarinum* cells because of their thick cell walls.

The time courses of the radiolabelled isotopes in Chapter four showed that it took much longer for the isotopes to reach a maximum level in the pellets of *C. submarinum* cells

when compared to *D. parva* cells. This suggests that the thick cell wall acted as a barrier to the isotopes.

The internal pH of both algae was seen to be maintained at a more neutral pH than the external pH of the media (Figure 4.3). The internal pH of the *C. submarinum* adapted cells was found to be slightly more extreme than adapted *D. parva* cells. These results contradict the theory that the thick cell wall of *C. submarinum* was offering greater protection to the cells. If this was so then it would have been expected that the internal pH of the *C. submarinum* cells would maintain a more neutral internal pH. It may be possible that *C. submarinum* cells can function normally over a range of internal pH values and the cells do not therefore require to maintain a rigid neutral internal pH. A second measurement of internal pH that contradicted the protectiveness of the *C. submarinum* thick cell wall was the internal pH value for the pH 5.5 shocked cells, which showed that after the three hour incubation period *C. submarinum* cells had a significantly lower value than the *D. parva* cells suggesting that the *C. submarinum* were more susceptible to changes in external pH.

The membrane potential of both algae was found to decrease in acidic external pH and an increase in alkaline external pH. The membrane potentials for *C. submarinum* were found to be greater than those measured for *D. parva*, this may be due to the negative charges present in the cell wall of *C. submarinum*.

The final evidence to support the hypothesis that the thick cell wall of *C. submarinum* offered greater protection to the cell from external changes is drawn from the enzyme activity results (Figures 5.1, 5.2, 5.3, 5.4). Enzyme activity was measured from both pH adapted cells and pH shocked cells. The pH shocked cells were resuspended in fresh medium for three hours before the activity of the enzymes was measured. Cells that were adapted to their external pH were resuspended in fresh medium of the same pH and the enzyme activity measured immediately. The enzyme activity in the shocked cells was found to be much higher than in the pH adapted cells. The vast difference in activity between the shocked and adapted cells was concluded to be a result of the difference in the length of time cells were resuspended in fresh medium before the activity measurements were taken (section 5.3). For all of the enzymes, except fumarase, the enzyme activity in the pH shocked cells was found to be higher in *D. parva* cells. This is probably because the thick cell wall of the *C. submarinum* cells protected the cells from the changes in external medium and thus it took longer for the cells to respond to the fresh media. In this instance the cell wall was actually inhibiting cell metabolism.

One area of great interest from this PhD study is the lack of enzyme activity found in *C. submarinum* cells for enzymes in the glycerol cycle (Sections 5.2.3, 5.2.4) The glycerol cycle in *Dunaliella* spp. has been well documented and the fact that *C. submarinum* produces glycerol has also been documented. Very little enzyme activity was found in *C. submarinum* for two of the glycerol cycle enzymes, glycerol-P dehydrogenase

(involved in the synthesis of glycerol) and dihydroxyacetone reductase (involved in removal of glycerol). If the glycerol cycle is active in *Chlorococcum*, it would be expected to find activity for these enzymes. No activity of these enzymes may be a result of the enzymes becoming damaged in the preparation of the assay. It may also be possible that *C. submarinum* does not produce glycerol by the same cycle as *Dunaliella* spp. It would be of great interest to study this problem further. This might be achieved by preparing the cell free extract using a different method or studying more enzymes in the cycle. It may also be prudent to grow the *C. submarinum* cells in medium with higher salt concentrations for several generations so that the cells have become adapted to the external salt levels and they are therefore healthier than those recently resuspended in altered medium and therefore actively producing glycerol.

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CHAPTER SEVEN

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Appendices

Appendix A

Dunaliella Growth Medium

Compound	Amount in 1 l
0.5 m Na ₂ SO ₄	48 ml
4.0 M NaNO ₃	1.25 ml
2.0 M MgCl ₂ 6H ₂ O	10 ml
1.0 M CaCl ₂ 2H ₂ O	10 ml
2.4 M MgSO ₄ 7H ₂ O *	10 ml
0.1 M NaH ₂ PO ₄	1 ml
4.0 M KCl **	2.5 ml
1.0 M Tris - HCl pH 7.6 ***	20 ml (20 mM) or 100 ml (100 mM)
1.5 mM FeEDTA pH 7.6 ***	1 ml
NaHCO ₃ (solid)	1 g
NaCl (solid)	87.66 g
Supplements	1 ml

Supplements

185 mM H ₃ BO ₃	0.02 mM CoCl ₂
7.0 mM MnCl ₂ 4H ₂ O	0.0002 mM CuCl ₂
0.8 mM Zn Cl ₂	

- * The Hydration level of the compound should be taken into account.
- ** Do not store Na₂SO₄ or KCl in the cold room; they come out of solution.
- *** Both Tris and FeEDTA must be altered to give pH 7.6: Concentrated HCl for Tris and NaOH for FeEDTA.

- 1) To 0.75 litres distilled water add the solid NaCl.
- 2) Add all the nutrient solutions except for NaHCO₃.
- 3) Check pH and adjust to pH 7.5 with HCl or NaOH.
- 4) Make up to 1 litre with distilled waster and add NaHCO₃.
- 5) Dispense medium into 1 litre duran bottles.
- 6) Autoclave for 20 minutes at 15lbs in⁻¹ pressure.

Appendix B

Chlorococcum Growth Medium

In 100 ml deionised water

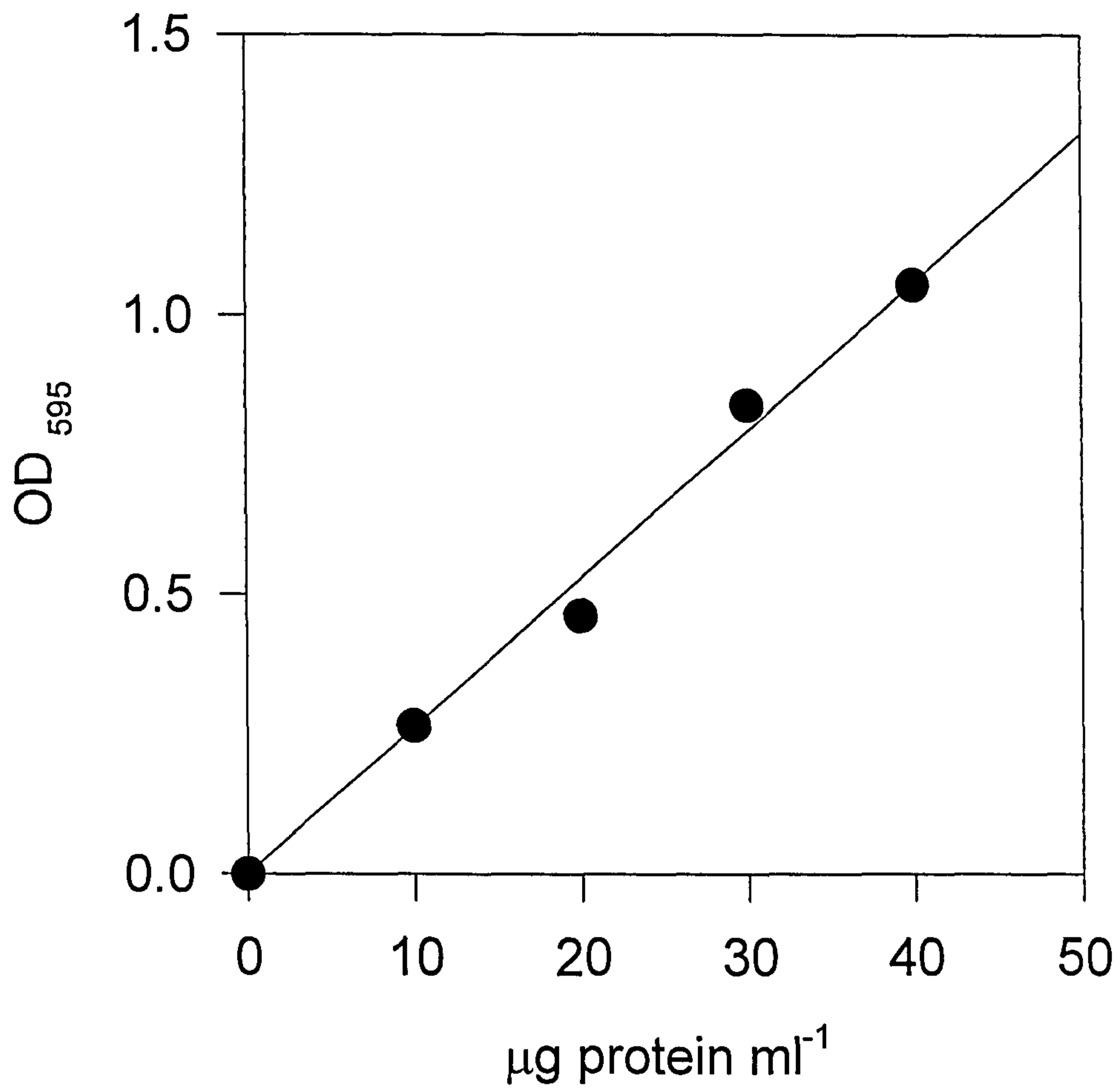
CaCl ₂ 2H ₂ O	3.7 g
MgSO ₄ 7H ₂ O	3.7 g
NaHCO ₃	1.3 g
K ₂ HPO ₄	0.9 g
NaNO ₃	8.5 g
(NH ₄) ₂ HPO ₄	2.3 g

To prepare the medium (1 litre) as follows:

- 1) Put 900 ml of deionised water into a 2 litre flask, add 14.6 g of NaCl (final concentration 0.25 M).
- 2) Add 1 ml of each of the above stock solutions.
- 3) Add 1 ml FeEDTA, 1 ml supplements and 20 ml Tris buffer pH 7.6 (all in Appendix A -*Dunaliella* growth medium).
- 4) Adjust pH to 7.5 and make up to 1000 ml with deionised water, mix well.
- 5) Autoclave for 15 min at 20 lbs in⁻¹.

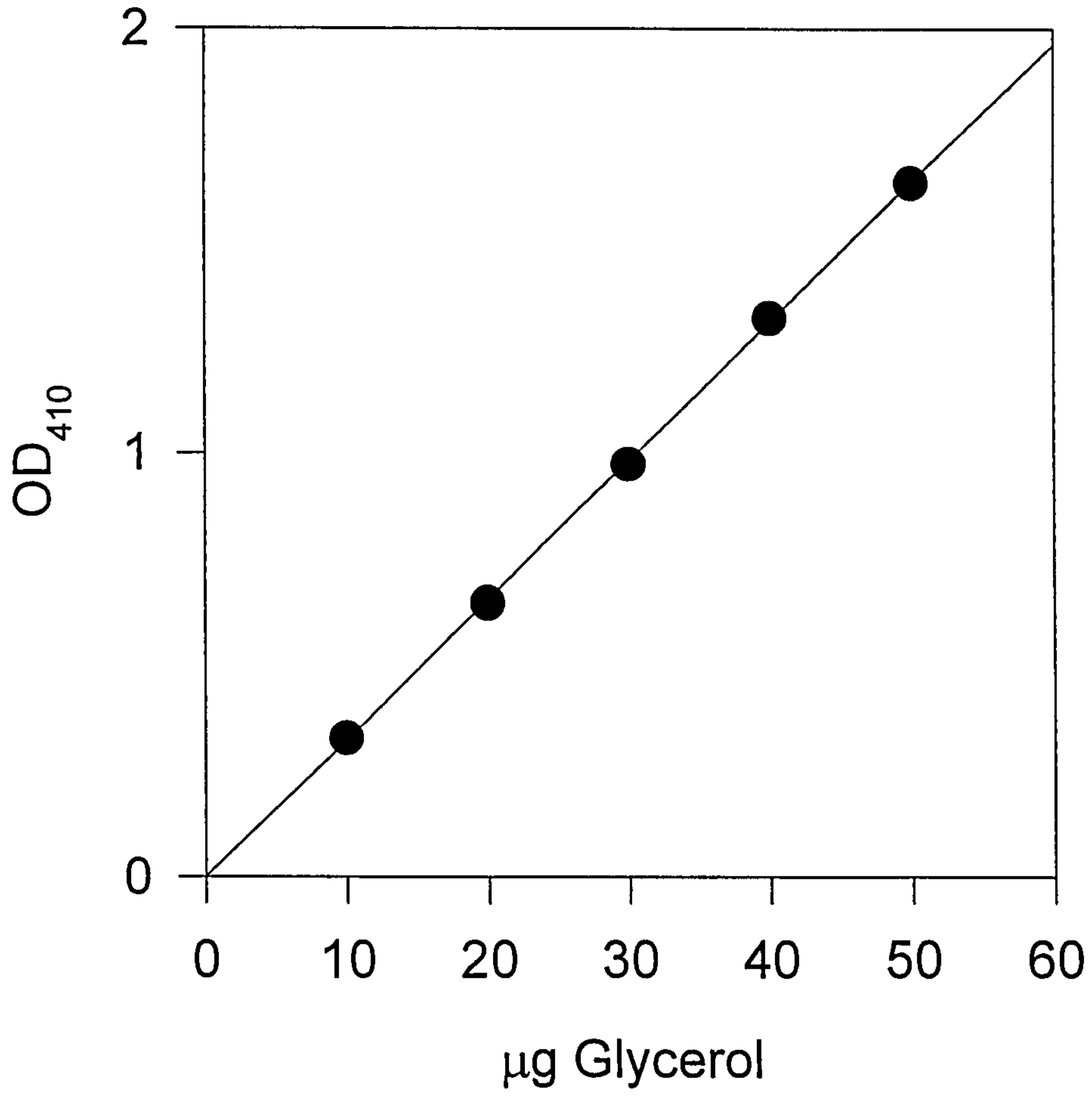
Appendix C

Standard Protein Concentration Curve.



Appendix D

Standard Glycerol Concentration Curve.



Appendix E

TEM preparation

Prepared by Mr John Proctor in the Electron Microscope Unit at Sheffield University.

Dunaliella parva 1.5M NaCl

Chlorococcum submarinum 0.25M NaCl

Soln. A

25% Glutaraldehyde 24 ml

Distilled Water to make 100 ml

Soln. B

Soln. A 50 ml

Saltwater 50 ml

(Saltwater: *D. parva* - 8.76g/100 ml: *C. submarinum* - 1.46g/100 ml)

Soln. C

Distilled Water 50 ml

Saltwater 50 ml

Soln. D

2% Osmium Tetroxide 50 ml

Saltwater 50 ml

The specimens were centrifuged to a pellet and then fixed in Soln. B for 90 minutes at room temperature.

This was followed by washing three times in Soln. C with 30 minute intervals.

Secondary fixation was carried out in Soln. D for 60 minutes at 4°C.

The specimens were dehydrated in a graded series of ethanol with steps of:

75% ethanol 15 minutes

95% ethanol 15 minutes

100% ethanol 15 minutes

100% ethanol 15 minutes

100% ethanol dried over anhydrous CuSO_4

Propylene Oxide 15 minutes

Propylene Oxide 15 minutes

The specimens were infiltrated in 50/50 Propylene Oxide/Araldite resin overnight.

The specimens were further infiltrated in full strength Araldite resin for 6-8 hours after which they were imbedded in fresh Araldite resin in gelatin at 60°C for 48 hours.

Thin sections, approximately 90 nm thick, were cut on a Reichert Ultracut E ultramicrotome, stained with 3% Uranyl Acetate in 59% ethanol for 50 minutes followed by staining in Reynold's lead citrate for 2 minutes.

The sections were examined on a Philips CM10 transmission electron microscope at an accelerating voltage of 80 Kv. Micrographs were recorded on Agta Scientia 23D516 electron microscope film.