ALGAL BIOTECHNOLOGY AND THE BENEFICIATION OF SALINE EFFLUENT WASTES

THESIS

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CONTENTS

ABSTRACT

Saline deterioration in the South African public water system has been documented and disposal of brine wastes has been identified as part of the problem. The broad aim of this research programme was to undertake an initial technical study to evaluate the feasibility of integrating algal biotechnology into a disposal function for these wastes. A demonstration of utility in the form of products and waste treatment could produce a beneficiation of saline effluents and provide incentives necessary to deal with the disposal issue.

The study attempted to demonstrate a synthesis between the two main thrusts in algal biotechnology that have produced large-scale practical applications - stable, predictable algal production in saline media and the cost effective High Rate Oxidation Ponding (HROP) process for incorporating algal production into a waste treatment function. Tannery organic saline effluents and the biotechnology of *Dunaliella salina* culture producing β-carotene were chosen as paradigms for the study.

1. The alga was shown to grow in certain tannery effluents producing enhanced biomass yields compared to defined inorganic medium cultivation. The potential for amino acid or protein supplementation of defined culture media was noted.

2. A reduction in organic load simultaneous with the growth of *D.salina* was recorded in laboratory-scale simulations of the HROP process. Rates similar to the fresh water HROP equivalent were demonstrated.

3. These results suggested the uptake and storage of organic nitrogen by *D.salina*. The consequent inhibition of β-carotene accumulation by the organism presented a potentially insurmountable obstacle to the feasibility of β-carotene production in this medium. Uptake and release of organic compounds, previously demonstrated in phytoplankton and other micro-algae, was confirmed in this study for *D.salina*. The evidence acquired indicated the internalization of both glycine and bovine serum albumin. An ultrastructural study demonstrated mechanisms by which this process might occur.

4. The release of substantial quantities of glycerol was shown. A mechanism whereby *D.salina* may use this to regulate ammonia availability via control of its associated bacterial population was observed. Glycerol release was identified as presenting an application in treating refractory organic wastes, such as secondary sewage sludges, by elevating C:N ratios. This could demonstrate a significant utility for brine waste impoundments.

5. A multistage production process was proposed to deal with the problem of β-carotene inhibition by separation of the growth and metabolite accumulation functions into separate unit operations. It was shown in this study that the stress of nitrogen deficiency combined with high salinity provides for effective β-carotene

accumulation under the conditions of low illumination that pertain in dense cultures. Subjected to these conditions effluent-grown cells show delayed but unimpaired β-carotene accumulation.

6. A role for the plant hormone abscisic acid in mediating the stress response was demonstrated in *D.salina*. Fluorescence induction studies suggested the presence of a signalling process forming part of a sensitivity control mechanism. Stress induction of β-carotene accumulation could occur through four clearly defined stages. Potential was identified for using this response as a physiological probe for monitoring and regulating the stress induction process.

7. The multistage processing concept requires effective algal cell separation technology. The use of cross-flow ultrafiltration and diafiltration with a polyethersulfone tubular membrane system was demonstrated as an effective process for the recovery and washing of *D.salina*. Cell concentrates were produced in a viable form.

8. Process designs incorporating the findings of the research programme are presented demonstrating how effluent and organic waste treatment functions may be combined with the production of *D.salina* and its products. Application of the multi-stage processing concept to β-carotene production in a defined medium process was identified as offering a potential four-fold yield enhancement. This could have a significant impact on a high cost, marginal algal biotechnology process. Aspects of novelty have been claimed in provisional patents applications.

A provisional demonstration of the feasibility of *D.salina* production in tannery effluent indicates that algal biotechnology may provide a utility for, and hence the beneficiation of saline effluent wastes.

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FIGURES

TABLES

ABBREVIATIONS

ABA	= abscisic acid
ASW	= artificial sea water medium
Aq	= aqueous
BA	= benzyladenine
BOD	= biological oxygen demand
Bq	= Becquerel
BSA	= bovine serum albumin
CCAP	= Culture Collection for Algae and Protozoa
CFF	= cross-flow filtration
CFMF	= cross-flow microfiltration
CFUF	= cross-flow ultrafiltration
COD	= chemical oxygen demand
D	= dilution rate
DO	= dissolved oxygen
dpm	= decay per minute
DWA	= Department of Water Affairs
EM	= electron microscope
GA	= gibberellic acid
GC	= gas chromatography
GC-MS	= gas chromatography mass spectrometry
ha	= hectare
HPLC	= high performance liquid chromatography
HROP	= high rate oxidation pond
IAA	= indole acetic acid
LIRI	= Leather Industries Research Institute
LMH	= litres per meter per hour
Me	= methanol
mS	= milli Siemens
MVAL	= mevalonolactone
NATSURV	= National Industrial Water and Waste-water Survey
NMR	= nuclear magnetic resonance
NTU	= nephelometric turbidity units
PA	= phaseic acid
PV	= permanganate value
PWV	= Pretoria-Witwatersrand-Vereeniging complex

SDS	= sodium 1-dodecanesulfonate
SDW	= solid dry weight
SEM	= scanning electron microscope
SS	= suspended solids
TCA	= trichloroacetic acid
TDS	= total dissolved solids
TDIS	= total dissolved inorganic solids
TKN	= total kjeldahl nitrogen
TLC	= thin layer chromatography
Vmax	= maximum utilization velocity
WRC	= Water Research Commission
WTC	= Western Tanning Company
10E	= exponential
μ	= specific growth rate

CHAPTER 1

INTRODUCTION

ALGAL BIOTECHNOLOGY AND THE SALINITY PROBLEM IN SOUTH AFRICA

Summary.

South Africa is faced with a serious problem of high salinity levels in the public water system and the situation is considered to be one of continuing deterioration. The various causes and effects of this problem are broadly documented. Appropriate preventive measures identified include dilution and the point source segregation and desalination of industrial saline effluents. This produces a highly saline brine reject stream which will require disposal in a manner that does not cause further environmental damage. Technology appropriate to deal with this disposal problem is poorly developed and this has been partly responsible for retarding the implementation of salinity control measures. It is proposed that the recovery of economic value from desalination brines would provide incentives and thus improve disposal feasibility. Various uses for desalination brines are enumerated, including saline algal biotechnology. An evaluation of the potential of algal culture in saline effluent brines forms the basis of this enquiry.

1.1. INTRODUCTION.

1.1.1. Salinity - The Problem.

Salinization, the accumulation of dissolved inorganic salts, is regarded as the single most serious threat of pollution facing the public water system in South Africa (Commission Report,1970;Department of Water Affairs - DWA,1986;Stander,1987). The Commission of Enquiry into Water Matters (1970) found that the mineralization of some of the Republic's rivers had reached such proportions that energetic investigation and action had become imperative at a national level. The viewpoint determining policy in the Department of Water Affairs is that the quality of many of the country's water resources is deteriorating rapidly and that the situation will get worse. Salinity is identified as the most problematic cause of this deterioration and as a result, quality may become a more important factor than quantity in determining the future availability of water, particularly in the interior of the country (DWA,1986,1990;Stewart Sviridov&Oliver,1988a). In 1990 a DWA official reported that the position remains one of continuously increasing salinities and that water supply quality management strategies and pollution control measures tend to conceal this underlying trend (Foster,1990). This

situation must be viewed against the general forecast that the demand for fresh water will exceed availability in the Vaal River supply area by the year 2025 (DWA,1989a).

Salinity is one of the most insidious and difficult to treat of environmental problems and it may have both natural and man-made causes.

1.1.2. Geological Salinity.

Geological formations such as the Karoo shales, are well impregnated with salts and when periodically inundated with flood waters are leached, and with an elevated water table may give rise to highly saline base flows (Bond,1946;van der Merwe,1962;Botha,1984;Schultz,1988;Allanson *et al.*,1990). Evaporative losses and flow reduction in rivers due to upstream diversion or drought conditions will likewise raise salt levels.

Salinity is also the result of human productive activity - agricultural, urban and industrial, and is the inevitable result of water usage, especially where recycle plays an important role in the overall water economy. It is the inevitable consequence of increased abstractions, consumptive use and return flows (Foster,1990).

1.1.3. Agricultural Salinity.

In agriculture, dry land farming results in an accelerated release of natural salts from soils which are then leached to the water system. Nearly all irrigation water also contains some salt which percolates to the water table and makes it increasingly brackish. Continuous irrigation, with evapo-transpiration of saline water, results in the accumulation of salt in the upper soil layers which can seriously affect plant growth and crop yields. In its final stages this will result in irreversible damage to the soil structure (Johl,1980). In addition, fertilizers and pesticides may also be contributing factors where run-off and seepage will result in elevated salinities for downstream users (Stander,1987).

This problem has been with mankind since the beginnings of civilization, affecting the Sumerians whose first highly organised culture, built about 3000 BC, was based on the irrigation of lands around the lower reaches of the Tigris and Euphrates Rivers. It has been suggested that salinization, due to continuous irrigation, led to declining productivity, an undermined economy and finally to the collapse of their cities, and mankind's first great civilization, around 2000 BC. While the ruins of this system are still evident, the lands have remained desolate and unproductive to the present time (Sinnigen&Robinson,1981).

The history of numerous major irrigation systems in modern times follows this pattern and Johl's 1980 review covers case studies from the USA's Imperial and Coachella Valleys drawing water from the lower Colorado River to Pakistan's Indus Plain, where over 20% of the land in this huge irrigation scheme was classed as severely affected. In less than a generation the Aswan High Dam in Egypt is causing salinization of cultivated soils which have for millennia been flushed by the annual flooding of the Nile River.

In South Africa irrigation, especially in the dry areas, is also beset with this problem with a number of schemes completely failed and others seriously affected (van der Merwe,1962;DWA,1986;Allanson *et al.*,1990).

I.1.4. Industrial Salinity.

Industrial and urban activity can result in the development of a pattern of salinization from its wastes, comparable in extent to that in agriculture, where rising salt levels are not only a threat to downstream land, but also adds an unacceptable cost to domestic and industrial users (Heynike,1981,1987; Heynike&McCulloch,1982). This includes the direct discharge of point source industrial and sewage treatment effluents, saline underground water pumped by the mines and run-off and seepage from industrial and urban areas, including mine dumps and atmospheric deposition (Jones *et al.*,1989).

The Pretoria-Witwatersrand-Vereeniging (PWV) conurbation complex provides a case study in industrial salinization, with a long history of problems plaguing the supply of fresh water (Laburn,1979; Herold&Triebel,1990). The upper Klip River Valley and Zuurbekom wells, which supplied the early development of gold mining on the Witwatersrand, were soon to prove inadequate and were eventually abandoned due to seepage contamination. The Vaal Barrage was constructed in 1922 and then the Vaal Dam in 1938, but these supplies have also proved inadequate and are now augmented from inter-basin transfers from as far afield as the Tugela, Usutu and soon the Orange Rivers. One of the major problems is that the Vaal tributaries which drain the Witwatersrand, the Klip and Zuikerbosch Rivers, discharge their saline polluted waters above the Barrage intake. To overcome this problem the Zuikerbosch Water Purification Works intake was built upstream of these discharge points in 1949. Soon, however, saline water was being sucked upstream which led in 1969 to the construction of a pipeline, and in 1983 a canal, to feed fresh water directly to this intake from the Vaal Dam without entering the Vaal River water course.

Given the situation of rising demand, a high priority is placed on water conservation and recycle in order to meet current, and future estimated off-take requirements (DWA,1986,1989a). Rand Water Board system operating rules have been aimed at minimizing spillage of water from the Vaal Barrage. Ahout one third of the effluent emanating from the PWV complex drains back to the Vaal River, its primary water source (Herold&Triebel,1990). In the case of the Southern PWV, 64% of water supplied is returned as effluent with nearly 300mg.L^{-1} total dissolved solids (TDS) added in each cycle. The annual average TDS level at the Vereeniging intake had risen over 30 years from less than 180mg.L^{-1} in 1949 to over 600mg.L^{-1} in 1980. Peak flows of around 1000mg.L^{-1} have been recorded in recent years at the Vaal Barrage against recommended targets of 250-300mg.L^{-1} TDS (DWA,1986). South African Bureau of Standards' recommended level for potable water is 70mS.m^{-1} (equivalent to 350-500mg.L^{-1} TDS), with maximum allowable limits of 300mS.m^{-1} (equivalent to 2000mg.L^{-1} TDS) (DWA,1986). With the exception of cotton, wheat and groundnuts, most commonly irrigated crops have maximum salinity tolerance levels of around 600mg.L^{-1} TDS and below. Tobacco may not be irrigated with water exceeding 25mg.L^{-1} TDS. This indicates serious consequences for

downstream irrigation use of Vaal River water. Heyneke (1981,1987) has calculated that every 1mg.L^{-1} increase in TDS costs urban and industrial users about 0.11cm^{-3}, resulting in an annual cost, at estimated rates of increase, of about R250 million. The accuracy and conservative values of these estimates has been questioned and reviewed in a subsequent report commissioned by DWA (Steffen Robertson&Kirsten,1989;DWA,1990).

The salinity problem is not confined to the PWV area. The average TDS for the Buffalo River catchment below King Williams Town rose from 128mg.L^{-1} in 1964 to 994mg.L^{-1} in 1983. Two factories alone (a tannery and a textile works) contributed 88% of the non-natural salt load entering the Laing Dam. Similar trends, resulting from industrial pollution, have been measured in the Berg River, in a number of Natal rivers and elsewhere in South Africa (Hart,1982;DWA,1986;Allanson *et al.*,1990).

1.1.5. Action.

It was, in part, against the background of these trends that a broad based co-operative research and remedial strategy was recommended by the Commission of Enquiry into Water Matters (1970). This covered a wide range of issues including the conservation of existing water supplies, re-use of water (recommendation 12), desalination (recommendation 36), prevention of pollution (recommendation 13), co-ordination of water planning (recommendation 44) and the co-ordination of water research (recommendation 45).

The Water Research Commission was established in terms of the Water Research Act in 1971. A significant part of its research expenditure has been directed at the salinity problem. In 1975 a programme was undertaken to develop a hydro-salinity model to simulate the movement of water and salt through the Vaal River/PWV complex, and to enable a treatment of the complicated interdependence of factors necessary to predict the outcome of any course of remedial action (Herold,1980,1981;Stewart Sviridov&Oliver,1985). Subsequent application reports on the blending option have appeared (Stewart Sviridov&Oliver,1988 a,b,c;DWA,1990). On the basis of these findings a blending scheme was identified as being the most cost-effective and ameliorative measure (Herold&Triebel,1990). Side stream demineralization, the removal of saline mining effluent and enforced desalination of other industrial effluents were also considered. In the blending scheme, fresh water is drawn from the Vaal Dam and blended with downstream water to even out salinity peaks and maintain a target of 300mg.L^{-1} TDS. Release of water from the Vaal Barrage is caught in the Bloemhof Dam, which feeds irrigation users including the Vaal-Hartz Scheme. Du Plessis (1984) has identified the unit cost associated with mineralization as being very much smaller for irrigation than for urban use. Nevertheless, the disbenefits of increasing soil salinity for downstream users will accumulate in subsequent years.

Herold and Triebel (1990) warn that too ambitious a blending target cannot be maintained indefinitely without incurring a severe penalty in terms of system yield, given anticipated growth in water demand for the PWV area over the following decades. The DWA regards the dilution and blending option as a short-term or temporary solution for handling the problem of saline effluent flows. The process is inherently wasteful of fresh water and

depends on adequate supplies which are not assured. It favours, rather, the application of control measures aimed, in the long term, at the source of saline pollution (Stander, 1987). The advantages of eliminating pollutants at point source are well documented (Hart *et al.*, 1987).

1.1.6. DWA Policy on Salinization.

In determining water policy, the DWA has identified the following as some of the reasons for the increasing salinization of the country's water resources (DWA, 1986; Stander, 1987).

1. The TDS standard for effluent returns is too lenient and too simplistic;
2. Previous water pollution control legislation and policies were not strict enough;
3. The cost of fresh water is too low;
4. A lack of co-operation by effluent dischargers to meet legal requirements;
5. Irresponsible and negligent attitudes of effluent dischargers;
6. An ignorance of the realities of a worsening situation and short term planning by effluent dischargers;
7. The relatively high cost of desalination technology;
8. A lack of private initiative to address a common effluent problem on a joint or regional basis.

In view of a general decline in the quality of water resources, with the greatest concern being increased salinity, the Water Act and Water Standards were amended in 1984. Section 21 of this Act established the requirement that water used for industrial purposes must be purified before its discharge. A maximum increase of $75 mS.m^{-1}$ above that of the intake water is generally applicable. An upper limit of $250 mS.m^{-1}$ for seepage from industrial or mining areas has been set. The problems of diffuse sources of pollution and the handling and disposal of saline effluents by means other than discharge to the public water system are covered in section 22. Section 24 empowers the Minister of Water affairs to direct the restriction or termination of the manufacture, marketing or use of any substance which in his opinion could cause water pollution. This provision could be applied to reduce the quantities of chemicals and salts added in the manufacturing process (Stander, 1987).

The following guidelines have been set out to cover the point-source producers of saline effluents (DWA, 1986; Stander, 1987):

1. The principle that applies is that the "polluter pays";
2. Effluent purification must be considered an integral part of the industrial process;
3. Salinity control measures should be applied at the source of the salinity problem;
4. Long term or permanent solutions should be implemented by the effluent discharger;
5. As desalination technology becomes available and commercially feasible, industry will be required to use it to remove salts from effluent streams.

1.1.7. Desalination.

The 1970 Commission of Enquiry into Water Matters also identified desalination as a research goal to be pursued for the expansion of existing water supplies, the prevention of point source pollution and to increase the number of re-use cycles of water by industry. The WRC has actively funded research in this area which has resulted in the local development of polysulfone reverse osmosis and cross-flow ultrafiltration membranes at the Polymer Institute, Stellenbosch University. This technology has been successfully commercialised and practical applications of the system have been demonstrated for power station boiler feed water demineralization and the desalination of mine waters (Anon,1982;Botha,1984;Stewart Sviridov&Oliver,1986;Anon,1987;Buckley *et al.*,1987;Hart *et al.*,1987). A preliminary costing of large scale side stream reverse osmosis desalination of Vaal River water has been undertaken (Stewart Sviridov&Oliver,1988a). Removal of particulates and colloidal organics has been identified as a necessary prerequisite for cost effective desalination of organic effluents (Neytzell-de Wilde *et al.*,1987). Here also, appropriate cross-flow microfiltration technology has been developed by the Pollution Research Group, Department of Chemical Engineering at Natal University and likewise subsequently commercialised (Treffry-Goatly *et al.*,1987;Groves *et al.*,1985;Neytzell-de Wilde,1987).

Desalination on a larger scale could be particularly appropriate in cases where high salt loads are contributed in relatively small volumes. Mine dewatering and mine dump seepage produces 25% of the annual salt load reaching the Vaal Barrage - about 50 000 tons salt. This is discharged in only 5% of effluent volume (Jones *et al.*,1989). In contrast, diffuse sources of salinity, which contributes 40% of salt tonnages, are contained in the total storm water run-off to the Vaal River making this a difficult source to treat. Industry and sewage contributes 35% of total salt load in 32% of total volume (DWA,1986). Industries identified where desalination could play an important role are power generation, chemical, iron and steel, pulp and paper, textile, mining, food manufacture and processing, and the tanning and hides and skins curing industries (Botha,1984;DWA,1986).

The DWA "Salts" project (DWA,1989b) surveyed sources of saline effluent in a number of Witwatersrand towns and cities conducted on an industry by industry and a product by product basis. The object was to identify and quantify industrial sources of inorganic salt loads to enable assessment of the feasibility of segregating the saline portion of the total effluent for separate removal to treatment facilities. The results, while provisional and not comprehensive, do show that a significant portion of the increased total salt load leaving the municipal areas in their effluents, can be isolated in a very small fraction of the total volume, indicating the feasibility of this general approach. In one example, 60% of increased TDS caused by the particular industries surveyed could be practically segregated in only 8% of their total effluent volume. Expressed differently for the whole town, this amounted to 10% of the total TDS added to the municipal effluent, and was contained in only 0.45% of their total effluent volume. The potential for further improvement was noted in the report, especially where incentives could be provided for improved manufacturing process housekeeping and the avoidance of dumping concentrated brines into bulk effluent discharge. Membrane desalination processes applied at point source could

further reduce the volumes of saline effluent to be handled.

1.1.8. Desalination Brines.

While the implementation of point source desalination will produce a substantial fresh water stream (80-90% of feed volume) and serve to reduce salt loads in the public water system, a smaller but more highly concentrated reject brine stream will also be produced. This will have to be disposed of, creating, in itself, a potentially serious problem. Whereas disposal sites for virtually all solid, toxic and other wastes can be found or engineered, sites for the effective disposal of brines have not been investigated sufficiently (DWA,1986).

Buckley *et al.* (1987) have commented that the lack of provision for adequate methods of brine disposal bas rendered the authorities responsible for pollution control powerless to enforce the desalination of saline effluents, despite legislation available to do so. Clearly resolution of the brine concentrate disposal problem will be required before point source desalination, on any scale, becomes a more attractive option than the current blending schemes.

Numerous methods have been suggested at various times for handling brine concentrates in inland areas (DWA,1986,1990;Stander,1987;Fijen,1988;Moolman,1990;Marquart,1990). These include:

1. The creation of salt water sinks or lakes that can be used for recreational purposes;
2. Co-disposal on solid waste disposal sites, mine tailing dams and ash dams at power stations;
3. Use in manufacturing processes such as ash quenching;
4. Deep well injection and dumping in disused gold mines;
5. Transport to the coast and discharge to sea;
6. The recovery of marketable products such as soda ash;
7. Incorporation into products such as fluting and liner board made by the paper industry;
8. Solar evaporation ponding with the production of a solid salt product;
9. Vapour compression evaporation and crystallization;
10. Irrigation directly onto land;
11. Enhancement of wetlands and agro-forestry;
12. Electricity production using solar brine ponds;
13. Saline algal aquaculture for the production of high value chemicals, biomass or fish.

1.1.9. Cost of Brine Treatment.

Principles that must govern any solution that is chosen are that it must not create more environmental problems than it solves. Seepage or saline sprays must not add to the diffuse saline load, and cost effectiveness of desalination and socio-economic cost benefits should be continually borne in mind (Muller,1990). In other words, the solution to the problem must not cost more than the cost of the problem. Verstraete and Huysman (1988), in identifying future research needs in environmental biotechnology, warn that many conventional

treatment techniques in current use provide an often perfect illustration of Murphy's Law - they merely transform one problem into another.

It has been suggested that local authorities will have to provide and operate central facilities such as evaporation ponds of sufficient capacity to absorb locally produced brines (from either direct desalination or the segregation of saline effluents). State involvement in the establishment and operation of regional facilities may be warranted. The full cost of these facilities should be recovered from the brine producers in keeping with the principle that "the polluter pays". This price could prove to be substantial. The cost of providing salt sinks has been examined for the DWA by Stewart Sviridov and Oliver (1985), and in a study on reverse osmosis desalination of water supply for lower Vaal River users, the cost of brine disposal was estimated at 10% of the total cost of plant. This figure would escalate several-fold if plastic pond lining was to be used to prevent ground water contamination (Stewart Sviridov&Oliver,1988a).

1.1.10. Incentive to Desalinate.

The incentive to desalinate saline effluents is clearly an integral part of the overall salinity problem. Since the principle of cost, in its widest sense, will dictate the most appropriate solutions to the problem, those technological options which offer the potential of recovery of value in some form or other, would warrant particular attention. Recovery of value could imply a wide range of options, but where the direct recovery of a by-product is implied, credits will be generated against waste disposal. This is a well established concept in improving the cost effectiveness of industrial processes (Hacking,1986) and was recognised at a WRC workshop where South Africa's future salinity research requirements were prioritised (du Plessis,1990). Alternative use for saline water and salinity control were identified as among those research needs with the highest priority second only to the existing programmes set up to assess future salinization trends. The final NATSURV report also identified similar research priorities in this field (WRC,1989b).

It is apparent, from the current trends documented above, that as fresh water availability declines and salinities rise, the point is being approached where separation of brine wastes will becomes essential. Development of appropriate technology, in advance of that event, is clearly indicated and would be the most cost effective strategy in the long term.

Moolman (1990) has warned, however, that novel ideas for handling the salinity problem should be treated with caution since, while theoretically sound, may be fraught with practical problems. Nevertheless, given the dimension of the salinity problem documented above, it seems equally clear that both creative and original thought is urgently required to be brought to bear on a problem for which there are few obvious or well proven solutions. A more detailed study of the feasibility of certain of the proposals that have been put forward is now warranted.

Concluding a detailed assessment of Department of Water Affairs thinking on the salinity issue, Stander (1987) warns that opting for easy solutions for present day problems will result in an enormous debt burden for future generations, for whom the cost of a solution could then be either exorbitant or prohibitive. "This would be an awesome burden of guilt for the administrator, the researcher and the industrialist."

Historical experience provides both a sober warning and a challenge to develop timeously, technology appropriate to the resolution of this problem.

1.2. SALINITY AND ALGAL BIOTECHNOLOGY.

1.2.1. Brine Disposal - Problem or Resource?

Effective technology for brine disposal and associated costs are major limitations in developing a long term strategy for the treatment of saline wastes. Since the amelioration of cost is, in part, at the root of the problem, it will be argued in this thesis that what is required, in defining research direction, is a change in mindset, where the beneficiation potential of brine concentrates be evaluated and by their treatment, to transform a problem into a resource.

The principle of value recovery is not new to waste management and while exercising caution with regard to over-simplification, wastes have, nevertheless, been regarded as something of a golden opportunity for biotechnology. Hacking (1986) points out that the boundary between wastes and by-products is arbitrary; where utility increases, a waste becomes a by-product. The term beneficiation describes the process whereby the creation or enhancement of the value of a resource takes place.

The value of wastes is determined by their opportunity costs and this increasingly acquires a higher negative value with rising disposal charges and penalties imposed by environmental legislation. As environmental awareness increases, rising waste treatment costs become more acceptable to society and industry (Verstraete&Huysman,1988). This can eventually tip the balance in favour of integrating waste treatment and some form of production, especially of microbial products. The beneficiation of wastes as by-products can have a decisive impact on the economic viability of industries producing them. Hacking (1986) has reviewed a number of biotechnology case studies where this principle applies.

1.2.2. Algal Biotechnology.

The broad objective of this research programme is to evaluate the feasibility of integrating algal biotechnology into a solution to the salinity problem and hence demonstrating a beneficiation potential for saline effluent wastes.

Algal biotechnology has developed rapidly in recent years (Chapman&Gellenbeck,1989), and the potential of aquatic photosynthetic production to deal with long term resource replacement and sustained, environmentally

sound, economic growth has been frequently identified (Oswald,1980;Soeder,1980,1986; Hall,1986;Simpson,1990). Photosynthesis is the most ahundant energy storing process on earth (Ben-Amotz&Avron,1989a), and the use of micro-algae (including Cyanobacteria) for the industrial production of biomass and speciality chemical products has long been recognised, and has recently been the subject of extensive review (Shelef&Soeder,1980;Richmond,1986a;Barclay&McIntosh,1986;Borowitzka& Borowitzka,1988a;Lembi,1988;Stadler *et al.*,1988;Cresswell *et al.*,1989).

Despite the enormous genetic potential of the micro-algae, and the observation that they can produce nearly all the products currently obtained from conventional land crops (Richmond,1986b), very few large scale outdoor algal production systems have been successfully established. Dubinsky (1986) has made the point that the occasionally observed algal water bloom has been the "philosopher's stone" of applied algology, offering an optimistic potential for pure culture which is difficult to reproduce under controlled conditions. Oswald (1988a) has commented that "there are no sustained axenic cultures out of doors in the real world."

Since the first major review of accomplishments in algal culture (Burlew,1953) through to the present time, the control of contamination and predation in algal cultures has been identified, among the many problems encountered, as one of the major limitations to be overcome for the successful future development of algal biotechnology (De Pauw&Persoone,1988;Ben-Amotz&Avron,1989a;Benemann,1989). As a result two main development thrusts can be identified in the field. In those applications where the purity of the algal product is of principle importance to the commercial success of the venture, research effort has focused on closed production systems and the manipulation of environmental selection factors. Where the production of mixed cultures with poor predictive control is acceptable, an open ponding approach has been followed.

1.2.3. Saline Production.

Pure cultures can generally be maintained in small-scale ponds and closed systems such as green houses (De Pauw&Persoone,1988) or tubular photobioreactors (Pirt *et al.*,1983;Vonshak,1988). The only really successful product-oriented, mass culture algal biotechnology systems, however, have been those where the exotic growth requirements of the algae in culture act as an environmental selection factor, ensuring the exclusion of competitors and the maintenance of a mono-species system. The two notable cases are the halophilic alga *Dunaliella salina* which grows in strong brine solutions (1.5-4M NaCl), producing β-carotene and glycerol (Ben-Amotz&Avron,1989a), and *Spirulina sp.* which are strongly selected for in an alkaline, saline environment (Richmond,1988a). *Spirulina* has a market as a high value health food, feed additive in specialist aquaculture rations and for the recovery of the pigments phycocyanin and phycoerythrin (Dubinsky,1986).

The selective nature of salinity is a well described ecological principle and Goldman and Horne (1983) have noted that in saline lakes, as salinity increases, there is a decrease in the diversity of organisms present, with an increase in productivity based on a few abundant species. In reviewing the current status and future prospects

of algal biotechnology, Richmond (1986b) has identified saline systems as offering the most immediate potential to progress towards economically feasible processes. He has identified significant economic advantages in cultivating the halophilic algae as salt-tolerant crops, utilizing land and brack water that is unsuitable for the production of conventional agricultural land crops. In many parts of the world sweet water is a scarce resource and it's availability for agricultural irrigation is rapidly becoming a serious global political issue (Anon. Time,Nov.1990).

1.2.4. Waste Water Treatment.

The use of algae in the treatment of fresh waste waters provides the other area of algal biotechnology which has seen significant practical development. Facultative algal lagoons have been in widespread use, as a part of the traditional approach to treatment processes and the final polishing of waste waters (Skerry&Parker,1985). The algal High Rate Oxidation Ponding process (HROP) has produced a major advance, providing a cheap, low technology alternative to these conventional, energy intensive aerobic systems for treating organic wastes. Aerobic biological processes for the treatment of organic effluents, including activated sludge and oxidation ditch systems, represents the largest and most widespread application of any biotechnological process (Horan&Eccles,1986).

The HROP process for treating sewage and animal wastes was pioneered by Oswald at the Sanitary Engineering Research Laboratory, Berkley, USA, (Gotaas&Oswald,1954;Oswald&Goluecke,1960) and the subject has been extensively reviewed (Shelef *et al.*,1980;Abeliovich,1986;Oswald,1988a). In this process an association is established between high populations of algae and heterotrophic micro-organisms, with photosynthetic oxygen production providing for the degradation of organics and the algae in turn, utilizing the inorganic nutrients and CO_2 produced. While the advantages of the process were at first thought to be limited to this mutual microbial association (Oswald&Gotaas,1955), it was subsequently realised that the algal component also contributes significantly to the degradation of low molecular weight compounds. They can be responsible for up to 50% of the heterotrophic uptake of organics from the system (Abeliovich&Weissman 1978;Abeliovich,1980,1986). Organic load removal in sewage operated HROPs can be in excess of 90% soluble biological oxygen demand (BOD), at retention times of 3-5 days (Oswald,1988a).

A polished effluent, suitable for discharge to the public water system, is produced with the removal of the algal-bacterial biomass, termed albazod (algal, bacterial, zoogloeal detritus). The albazod, however, has little value more than a cheap protein supplement and the cost of its separation from the waste stream has been a negative factor (Soeder,1986). The standing algal crop can reach 1.0-1.5g dry wt.L^{-1} in the HROP (Abeliovich,1980). Overall biomass yields can be around 150tons.ha^{-1}.$year^{-1}$ dry weight, of which the algal component accounts for 60%. This is close to the theoretical photosynthetic productivity limits for the system (Goldman,197;Shelef *et al.*,1980).

The precise control of algal species in these systems is not possible, with wide fluctuations in type following shock loading, seasonal changes and variations in effluent content. Abeliovich (1986) has noted a trend in species dominance, dependant on organic load, with highly polluted water containing *Euglena* and *Chlamydomonas* and in decreasing order of organic load *Scenedesmus*, *Chlorella* and *Micractinium sp.*

Nevertheless, Oswald (1988a) has described the HROP as the most efficient way known to fix solar energy in the form of biomass. Soeder (1986) sees the HROP, as applied to waste water treatment, offering the greatest advantage of all micro-algal biotechnologies with the potential to be exploited as a multi-purpose system. A number of other authors have drawn attention to the cost credits that are available where algal production is coupled to waste treatment (Grobhelaar *et al.*,1980). This could justify the use of otherwise expensive cell harvesting technology for the recovery of useful products (Benemann *et al.*,1980;Taiganides,1982). The incorporation of algal production into an already funded waste treatment process can deal decisively with the three factors that have been identified by Richmond (1986a) as most limiting in the further development of algal biotechnology; the costs of growth media, construction of ponds and the harvesting and recovery of micro-algae.

One of the few attempts at the linkage of waste treatment and algal production that has shown commercial promise is the use of organic wastes to induce artificial phytoplankton blooms in sea water where the biomass produced has been applied as a feed ration in aquaculture production (Ryther *et al.*,1975;Roels *et al.*,1976; Sandbank &Hepher,1980;Grobhelaar *et al.*,1980;De Pauw&Van Vaerenbergh,1983;Mitchell,1989). Soeder (1986) reports that the basic research has not led to large-scale practical application and De Pauw and Persoone (1988) have identified limitations associated with the process. However, substantial potential has been demonstrated in the cultivation of marine algae for feeding in specialist aquaculture production. Not only is a high value end product involved, but the absolute species purity of the algal feed is seldom of crucial significance making mixed culture acceptable. The cultivation of over 40 micro-algal species has been reported for feeding animals in aquaculture but De Pauw and Persoone (1988), nevertheless, note the persistent problem of a lack of stability and unpredictability where large-scale culture is attempted. The subject has been reviewed by Watson (1979) and De Pauw *et al.* (1984).

1.2.5. Integrating Salinity and Waste Water Treatment.

While the advantages of linking algal production and waste water treatment have been noted, an area that has apparently received little attention is the application of hypersalinity selection to the HROP treatment concept, and thus producing a synthesis of the two main thrusts of algal biotechnology which have yielded practical results - saline algal culture and the HROP waste treatment process.

The selective advantages of high salinity levels (>1.5M NaCl) in producing stable pure cultures of extreme halophiles such as *Dunaliella* has been demonstrated and, as already noted, is one of the few metabolite producing algal biotechnology processes to be commercialised in large (4000m^2) open ponds (Ben-

Amotz&Avron,1989a). The problem of costs and the economically marginal nature of this form of algal production has been noted (Borowitzka,1991) and will receive further attention below. The waste treatment linkage may not only deal decisively with the cost problem, offering the credits of cheap media, pond construction and effluent treatment, but the HROP also offers a well engineered process for the effective production of large quantities of algal biomass (Oswald,1988b). Both brine solutions and organics are available as problem production wastes, requiring treatment and presenting an opportunity for co-disposal in an algal culture process. The addition of the salinity factor offers advantages of control not available in the fresh water HROP - the regulation of predation and competition and the production of a predictable algal population from which products of value may be reliably derived.

While this is essentially a speculative consideration of the theoretical potential, Soeder (1986) has identified the development of the HROP into a multi-purpose algal production system, as a long term objective for algal biotechnology. Although the range of products currently available from the extreme halophiles may be limited, Richmond (1986c) has speculated that with the development of a system offering a stable and predictable crop cultivation approach to algal biotechnology, developments in the genetic manipulation of the micro-algae (see also Craig *et al.*,1988) could access a wider range of metaholites from this group of organisms. In this regard, saline algal culture could offer the potential production of a range of economically significant products currently obtained from terrestrial plants or from heterotrophic organisms cultured in expensive bioreactor systems. The biosynthesis of high cost speciality products is likely to provide the "research pull" on which future algal biotechnology will develop, since food production in the form of micro-algal biomass (with the possible exception of algal aquaculture rations noted above) still suffers from the problems of low product value that are common to other single cell protein projects (Hall,1986). Food production from waste is also faced with aesthetic objections (De Pauw&Persoone,1988).

It is apparent that a synthesis of saline algal production and waste treatment could provide a utility for waste brines and saline effluents, and given the production of useful products, possibly address the issue of incentives required for their treatment. In this sense algal production could constitute a beneficiation treatment by transforming a waste into a resource. However, the problems to be anticipated in realising this potential would not be of a trivial nature. Furthermore, despite optimism concerning the potential for linking effluent treatment and algal production via the HROP, Abeliovich (1986) has warned that notwithstanding significant efforts in the field, the process remains essentially experimental.

PART A:

ALGAL CULTURE IN SALINE WASTE

The broad objective of this research programme was to explore the feasibility of integrating algal production into a viable solution to the problem of saline effluent waste disposal. The basic question to be asked was whether halophilic algae can be successfully grown in industrially generated brines and produce products of value. With the demonstration of a beneficiation potential for saline effluent wastes, subsequent questions would concern the practicality of accomplishing this objective.

While it was accepted at the outset that the experimental programme would need to be product focused, an ideal outcome would be the demonstration of a broadly applicable, enabling technology for dealing with the wider environmental management issues of this particular problem.

Borowitzka and Borowitzka (1989) have generalized a development programme for algal biotechnology projects involving a number of distinct phases, the first of which concerns the evaluation of biological and technical feasibility. This they have termed the initial technical study. Chapman and Gellenbeck (1989) have also drawn attention to the thorough understanding of the biology of algal processes required before proceeding through the subsequent phases of scale-up evaluation. The reference frame for the programme that is reported here can be largely characterised as an initial study of technical feasibility. The final objective, accepting the successful demonstration of feasibility, would be the development of a process design as the end product of the programme. This would aim to integrate the relevant biological, technical and engineering details required to serve as a practical basis for subsequent scale-up evaluation of the programme.

1. ASSUMPTIONS.

The enquiry was premised by a number of assumptions which served to focus the subsequent investigation:

1. Of the brine disposal options that have been suggested in the literature, and detailed above, the least attractive is the dumping of saline brines in sinks, or any variation thereof, where salt will accumulate indefinitely and possibly create insoluble problems for the future;
2. In principle, the solution of choice should be linear, whereby salts are concentrated and ultimately recovered from brines and disposed of in a way that effectively removes them finally from the environment;
3. While high cost, energy intensive evaporation technology does exist to accomplish this task both rapidly and effectively, it is more likely that a "low technology" ponding and solar evaporation process would be cost effective and appropriate to the scale of the problem;

4. The simplest application of this process would be some form of linear cascade of ponds with a rising gradient of salinity, resulting in the production of a saturated brine solution from which salts can be crystallized;
5. It is unlikely that a universally applicable algal culture system will be found to be appropriate for all brine streams. While some form of pooling of different brine streams is to be anticipated, separate systems will probably be required for the various types of saline effluent;
6. Tanneries, as major producers of saline effluents, were identified as providing a potentially appropriate model to serve as a basis for this study.

2. RESEARCH HYPOTHESIS.

The above assumptions led to the formulation of the hypothesis on which the subsequent research programme was based:

> Tannery effluents and saline evaporation ponds, as used in the tanning industry for effluent treatment and disposal, constitute an unutilized resource, suitable for exploitation in the development of a halophilic micro-algal biotechnology production process.

3. EXPERIMENTAL PROGRAMME.

The basic questions identified as requiring answering first were those needed to validate the research hypothesis and establish a sound biological basis for the subsequent refinement of the process. The initial experimental programme was designed to answer the following questions:

1. Is the assumption valid that the tanning industry effluent treatment system provides an appropriate model for studying the linkage of algal biotechnology and brine effluent disposal;
2. Can saline tannery effluent be used as a growth medium for the culture of commercially important halophilic algae, providing a cheap alternative to costly defined-media culture systems;
3. Can the HROP be operated as a halophilic system, providing an effective process design for linking the treatment of saline organic effluent and the production of a halophilic alga in reasonably pure culture as its major product;
4. If the application of the HROP concept is found to be valid, what features or limitations of the process need to be identified (and require more detailed investigation) to facilitate the design of an operational process as the end product of the research programme?

CHAPTER 2

TANNERY EFFLUENT AND SALINE ALGAL CULTURE

Summary.

Tannery effluent was chosen as a paradigm for the study of algal culture in industrial saline effluents. The reasons for this choice are documented against the background of effluent treatment systems commonly in use in the industry. A case study in the evaporation ponding of saline tannery effluent is reported which served as the practical basis for the design of the subsequent experimental programme.

2.1. INTRODUCTION.

Tanneries have been described as producing the most polluting wastes of any industry (Tsotsos,1986). In South Africa some twenty tanneries process about 2 million hides annually, using approximately 600 000m^3 water, almost all of which becomes waste effluent (WRC,1989a). This study documented the average pollution loads in these tanneries (see table 2.1).

Table 2.1 The average pollution load contributed to waste water by South African tanneries (WRC,1989a).

	Specific Pollution Load. ($kg.hide^{-1}$)	Raw Waste Water Quality. ($mg.L^{-1}$)
COD	3.7	9700
TDS	7.7	19600
SS	0.8	1970
Chrome	0.1	120

Up to 70% of the TDS value can be total dissolved inorganic solids (TDIS) due to chlorides and sulphates used in the tanning process and equally importantly, as sodium chloride from salt cured hides.

The traditional method of preserving hides and skins is by "salt curing" - the saturation of the water content of the hide or skin with sodium chloride (Cooper *et al.*,1984a). This results in a reduction of moisture content of approximately 30% of the green hide weight and this mass loss is offset by the uptake of salt by the hide, to 14% of its final cured weight. About 8 litres of a near saturated brine is produced per hide during the curing process (Cooper *et al.*,1984b). Hides and skins may be stored in this state for years.

At the start of the tanning process, the salt is removed from the hide by soaking (producing Hide Soak Liquors), as a preliminary step to the unhairing and liming stages, processes in which salt can have a detrimental effect. The dissolved salt passes to the effluent with the hide soaks constituting about 20% of tannery water usage.

Cooper *et al.* (1984b) have reviewed a closed system approach for dealing with salt and saline waste water produced in the curing and tanning processes. A novel approach pioneered by the Leather Industries Research Institute (LIRI), Rhodes University, involves the replacement of salt by the use of antiseptics (Russell&Galloway,1981). This can reduce TDIS in the final effluent by about 30%. Numerous problems are, however, encountered with antiseptic use, including limited storage margins (approximately 8 days), which affects the traditional trade in salted hides. Biocides pass to the effluent affecting subsequent biological treatment processes. Boron used synergistically with the antiseptic has been found to rise to unacceptable levels in the environment (Rowswell, pers. comm.). A combined low salt/antiseptic treatment process has been developed at LIRI which allows effective storage of hides for periods of several months (Russell&Galloway,1977).

The preservation of hides in a refrigerated cold chain and the processing of fresh green hides to the wet blue stage, directly from the abattoir, are among other currently favoured alternatives to the use of salt curing.

2.2. TREATMENT.

Processes for the treatment of tannery effluents have been the subject of considerable study world-wide, and have been extensively reviewed (Constantin&Stockman,1977;Polkowski *et al.*,1978;Carre *et al.*,1983;Cooper *et al.*,1984a;Panzer&Komanowsky,1985;Tsotsos,1986;WRC,1987,1989a).

Traditional methods of disposing of tannery effluents include discharge to facultative lagoons, evaporation ponds, spray irrigation to land and discharge to the municipal sewer (WRC,1989a). The facultative lagoon is a well established process that can be used either with or without the pre-settlement of solids, producing an effluent of sufficient quality to permit recycling. Lagooning, for water saving in arid areas, has been found in archaeological excavations to have been in use by tanneries since at least biblical times (Sandbank, pers. comm.). Effective design criteria have been established for these systems more recently, and their detailed operation reported (Polkowski *et al.*,1978). Significant reductions in total kjeldahl nitrogen (TKN), suspended solids (SS) and permanganate value (PV) are accomplished by the process (Rowswell *et al.*,1984).

Due to environmental pressures, on-site pre-treatment is becoming increasingly important before final discharge to ponding systems, in order to deal with soil degradation, ground water pollution and odour problems (WRC,1989a). Where a physico-chemical pre-settlement of solids is practised, a chemical sludge is produced which requires disposal to landfill. These systems are currently under pressure from environmental authorities due to the toxicity of the leachate and lack of space availability. The potential for the oxidation of chrome III to highly toxic chrome VI form has been noted, and in certain European countries the disposal of tannery waste

to landfill has either been banned, or packaging in plastic bags is required.

The development of activated sludge and oxidation ditch processes over the past two decades has contributed considerably to the generation of recyclable effluents (Bailey,1977;Panzer&Komanowsky,1985;WRC,1987). Less problematic humus-type sludges are produced, and up to 90% of the influent organic load (BOD) can be removed in this way (Kiestra&Eggers,1986).

Disposal of the saline content of tannery effluent also presents a somewhat intractable problem. Where discharge to the municipal sewer is not available or permitted, evaporation ponding has been used as an additional step to facultative lagooning. The evaporation ponding process has been reviewed by Rowswell *et al.* (1984) and in principle involves the provision of sufficient ponding area to allow an evaporative loss of water, greater than the volume of effluent discharged by the tannery. Salts accumulate to saturation levels in the final ponds and the salinity gradient across the system is characterized by mixed cultures of halophilic bacteria and algae. All effluent is ultimately disposed of by evaporation producing, in effect, an environmentally closed system. The accumulation of sludge is a problem where pre-treatment is not practised. A number of these systems are in operation in South Africa.

More active intervention, to remove salt from tannery effluent, has also been considered (Schutte,1983). Neytzell-de Wilde *et al.* (1987) report an investigation by the Natal University Pollution Group, of a range of membrane desalination techniques for treating tannery and curing effluents, including electrodialysis and reverse osmosis. Ultrafiltration and hyperfiltration were used as pre-treatment steps for the raw effluent, but the generally unsatisfactory results were suggested to be due to the high organic load and colloidal fraction in these liquors. Reverse osmosis has, however, been successfully used in the desalination of wastes such as sewage where organics have been removed by pre-treatment (WRC Fish Water Flats project - final report pending).

The identification of a pre-treatment requirement for membrane based desalination has also contributed to shifting the focus of tannery waste processing back to biological systems for producing suitably low BOD effluents. A high rate biofilm trickle filter system for treating raw effluent has been designed by LIRI and installed at a Port Elizabeth tannery. Algal ponding has been used as a final polishing step for municipal effluent containing a tannery discharge at Napa, California (Ramani&Oswald,1975). The need to develop a high rate algal system for polishing final treated tannery effluents has been made apparent by the introduction of nitrogen load into the tariff formula for effluent treatment charges by certain municipalities in South Africa.

The future trend in handling the tannery salinity problem appears to lie in some form of pre-treatment followed by membrane desalination. However, as Schutte (1983) has pointed out, this does not dispose of the salt, but merely converts it into a more handleable form - a reduced volume of high strength brine.

2.3. TANNERY EFFLUENTS - A PARADIGM FOR THIS STUDY.

While the effluent problems of the tanning industry as outlined above are substantial, their relevance to this study is that they appear to offer an appropriate paradigm for a generalized evaluation of halophilic algal production in saline industrial effluents.

A number of criteria were involved in the choice of the tannery effluent ponding process as a basis for this study and are summarised below:

1. The tanning and curing industries are known producers of saline effluents, and disposal, especially of the saline component, has been a long-standing problem;
2. The construction and operation of evaporation ponds, similar to those which could be used for the handling of desalination brines, is a well established, traditional approach to saline effluent disposal in the tanning industry, and several ponding systems are in use in South Africa and available for study;
3. Pond construction and media costs have been identified as major factors limiting further development in algal biotechnology, and tannery waste treatment facilities could offer advantages in both areas;
4. Tannery effluents combine the features of high organic loads, heavy metal contamination and biocide content - all of which are to be expected from a combined saline effluent stream that could flow from an industrial area;
5. Problems to be anticipated in the practical application of otherwise sound novel ideas, contributed to the decision to use a "real life" reference as the basis of the study, in the first instance, rather than attempting to found, possibly far reaching conclusions, on the use of a defined synthetic system. The warning by Moolman (1990) has already been noted, of problems to be anticipated in theoretical studies based on novel solutions in this field.

The potential advantages of linking algal culture technology to the tanning industry's effluent treatment problems would include:

1. Photosynthetic oxygenation can offer significant savings in aerobic treatment processes where mechanical aeration is a substantial cost factor;
2. The HROP system is already well understood as applied to fresh water treatment. The development of a saline algal HROP system treating tannery effluent could lean heavily on this technology;
3. The removal of organic and inorganic nutrients could allow the final disposal of saline effluents by further membrane desalination, vapour pressure crystallization or by dilution in storm water without eutrophication risks;
4. The potential of a financial return in the form of algal metabolites would be beneficial for an industry for which waste processing has always consisted of a nett debit against production;

5. Odour abatement effects would be equally welcome to an industry which has faced continual environmental pressure in this regard.

2.4. THE WELLINGTON TANNING CO. - A CASE STUDY.

The programme reported here commenced with a preliminary study of an evaporation ponding effluent treatment system in operation at the Western Tanning Co.(WTC), Wellington RSA. The company had experienced problems with the operation of the ponding system and a consultation study was undertaken, and the findings presented, in an internal LIRI Technical Report (Rowswell&Rose,1990). A number of visits to the tannery were required over a period of time and several useful conclusions could be drawn from this investigation. A summary of the report follows.

2.4.1. Report.

The tannery has been in production at the same site for over a century. The existing effluent treatment system consists of a cascade of 15 ponds (figure 2.1), constructed and in operation since the mid-1960s, and designed to treat 270m^3 per day (13.6ha. surface area, 197 000m^3 capacity and varying in depth from 0.5-3M).

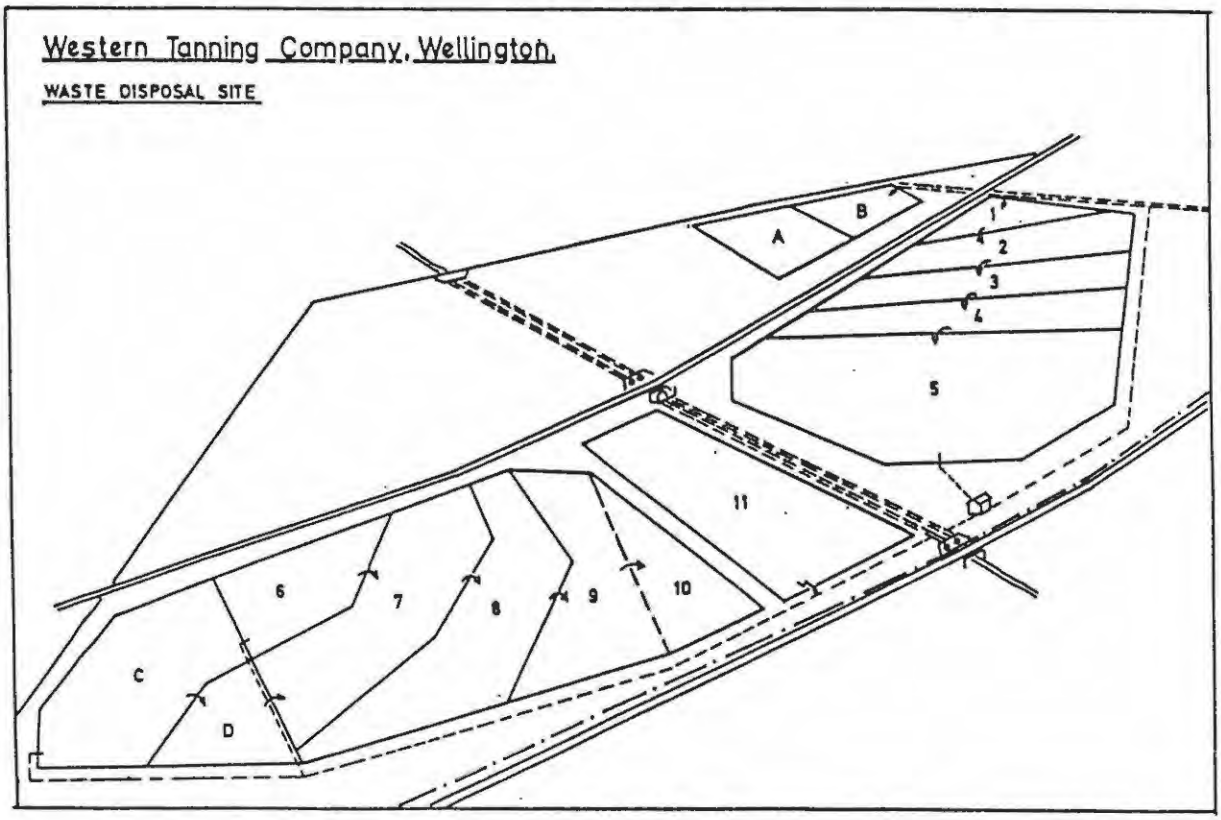


Figure 2.1 A plan view of the WTC evaporation pond system. All effluent and rainfall run-off from the WTC site in Wellington is received by this system. Liquid enters at pond 1, is pumped from pond 5 to pond 6 and the concentrate finally evaporated in ponds 10 and 11.

Following problems with sludge accumulation in ponds A and B, a Huni-Idronova physico-chemical pre-treatment system was installed during the mid-1980's where solids are settled by aluminium and polyelectrolyte flocculation and disposed to landfill. Licensing by the DWA for the operation of a closed site required the collection of all seepage and rainfall run-off from the entire 40 ha. site to be pumped to the ponding system. It was found that effluent production had increased at the same time to over 450m^3 per day, and while additional ponds C and D had been constructed, the system was not coping with the extra flow.

Meterological data for the area were updated and a water balance determined for the system from which it was calculated that an extra 10.74ha pond surface area would be required to handle the excess flow. Since site limitation and cost considerations would not allow an expansion to the pond system, a number of alternatives were considered and recommendations made.

2.4.2. Recommendations.

1. Annual rainfall run-off is nearly equivalent to fresh water usage by the tannery (94 000m^3 and 108 000m^3 respectively). It was proposed that all rainfall run-off should be pumped to a collection pond, and the tannery's fresh water requirements be drawn from that source. This together with the reuse of a certain percentage recycle of pond contents would place the system back into a substantial negative water balance.
2. A biological process such as an oxidation ditch as a primary treatment step producing a humus-type, instead of a chemical sludge, would have obviated, in part, the need for a licensed landfill and thus the ponding of all run-off which was the proximal cause of the problem.
3. It was noted that a programme of harvesting algae present in the pond system offers the advantage of removing N, P and K from the final pond effluent, thereby reducing the risks of eutrophication in downstream impoundments in the case of releasing clarified saline effluents. This also raised the prospect of a more active algal treatment process along the lines of the existing fresh water HROP system.
4. Also considered was the relocation of the brine concentrates to an unutilized 10ha system of ponds, approximately 2km from the tannery, where final evaporation and crystallization could take place. The potential for a controlled halophilic algal aquaculture operation in this system was noted.
5. Cross-flow filtration (CFF) systems were evaluated for the recycle of clarified pond water to the tannery and for harvesting algal biomass. This demonstrated a novel use for CFF and the results are reported in Part C.

2.4.3. Microbiological Evaluation.

The microbiological evaluation of the evaporation ponding system showed well stratified, aerobic and anaerobic populations. High influent sulphur content in the form of sulphides and sulphates is supplemented by protein degradation, which is accompanied by characteristic mercaptan odours.

The predominant bacterial population are the purple Archaebacteria, *Halobacterium sp.* - probably *H.halobium* (but species not determined) which gives the first ponds a characteristic bright red to purple colour (see figure 9.14a). These organisms are known to function as both photoautotrophs and heterotrophs as they move through the water column from anaerobic to aerobic and illuminated conditions. Their heterotrophic requirement for amino acids and other organic acids, sodium for their uptake and high sulphide levels are well supplied by the tannery effluent medium (Brock&Madigan,1988).

Photosynthetic and non-photosynthetic sulphur bacteria occur in large numbers and can be observed by microscopic examination to accumulate in bottom sediments. The following were tentatively identified using morphological descriptions from Bergey's Manual (Staley *et al.*,1989) - the Purple bacteria *Chromatium sp.*, *Thiospirillum sp.*, *Thiopedia sp.* and the Green bacterium *Chlorobium sp.*. At times these organisms appear to bloom and float on the surface as a white paint-like film of sulphur. A cycle of continuous sulphur oxidation and subsequent anaerobic reduction in bottom sediments appears to be in operation.

The latter ponds (6-11) are green in colour and support large population of algae. The predominant photosynthetic organisms are Cyanobacteria, *Spirulina sp.* (possibly *S.platensis*), and occur in large blooms which can be wind concentrated at the end of the ponds (see figure 2.2). The standing crop of predominantly micro-algal biomass in ponds 6-10 and C and D (4.5ha) was measured gravimetrically at 1.09Kg.m^{-3} solids dry weight (SDW) - equivalent to a total of about 100 tons across the system.

The predominant green alga is *Dunaliella salina* - other species also appear which were tentatively identified as *D.tertiolecta* and *D.parva* by comparing type strains from the Culture Collection for Algae and Protozoa (CCAP). *Dunaliella species* appeared in all ponds and exhibited a circadian rhythm, rising and falling through the water column during the day. At times, wind concentration would produce apparent blooms at pond ends. Wind concentration of *Dunaliella* in open ponds has been reported by Borowitzka *et al.* (1984).

Figure 2.2 Algal blooms and rafts of *Spirulina* on ponds at WTC, Wellington.

2.5 CHOICE OF ALGAL SPECIES.

Both *D.salina* and *Spirulina sp.*, as already observed above, are algal species with demonstrated industrial potential and are well studied micro-algal systems for biotechnological exploitation. Their prolific occurrence in the WTC ponds focused attention on their potential for further study within the context of the beneficiation of this waste.

A total value of approximately R200 000 for the standing crop of *Spirulina* in the pond system was calculated based on a current fishmeal price of R2000ton^{-1}. This is a conservative estimate since it was noted in promotional trade literature that Earth Rise Farms in USA are marketing a pure cultured dry powdered Spirulina product, as a speciality aquaculture feed, priced at \$15 000-\$19 000ton^{-1} (Henson,1988).

State-of-the-art algal biotechnology for the recovery of both glycerol and β-carotene has been recently reviewed by Ben-Amotz and Avron (1989a,1990) and Borowitzka and Borowitzka (1988b,1989). *D.salina* produces significant quantities of β-carotene, with a yield potential of up to 1ton.ha^{-1}.year^{-1} in pure culture systems (Ben-Amotz&Avron,1989a). While the market price current of β-carotene varies quite widely depending on the quality and formulation of the product, at a price of around R5 000kg^{-1}, gross returns of several million Rands are thought to be feasible (Benemann *et al.*,1987, and personal communications within the industry.)

A detailed evaluation of the feasibility of recovering value in the form of *Dunaliella* and *Spirulina* cultured in tannery saline evaporation pond effluents was identified as an appropriate biotechnological exercise. The problems to be anticipated in such an undertaking, however, are not trivial and due to the anticipated complexity and significant differences that exist between the two algal systems, it was considered appropriate to separate the experimental components of the *Dunaliella* and *Spirulina* studies. The evaluation of *Dunaliella* forms the basis of the programme reported here. The *Spirulina* work has been undertaken separately as an MSc programme within this laboratory and the work has been funded by a Water Research Commission Research Grant.

To further reduce complexity it was decided to concentrate initially on the recovery of β-carotene only from *D.salina*. Many of the findings would, nevertheless, be expected to be of relevance in an assessment of the feasibility of glycerol and protein production from the same source. The recovery of glycerol from *Dunaliella* has been previously reported (Ben-Amotz&Avron 1980;Chen&Chi,1981) and yields and cost benefit estimates could be calculated once cell production in a particular environment was established.

CHAPTER 3

THE PRODUCTIVITY OF *D.SALINA* IN TANNERY EFFLUENT

Summary.

The growth of *D.salina* was demonstrated in a range of tannery effluents indicating their suitability as production media. Enhanced cell production was recorded and the presence of a nitrogen storage mechanism was observed in effluent-grown *D.salina*. The effect of this on β-carotene production was noted. Comment is made on the possible involvement of organic nitrogen in this effect.

3.1. INTRODUCTION.

The nature of the final effluent produced in a particular tannery will depend, to some degree, on the product produced. This may be either partly processed "wet blue" chrome tanned hides or skins (which are traded in the wet form) or finished, dyed leather. A range of individual effluent streams are produced in the various steps of the tanning process. These have very different constituents, but, on average, the final effluents are sufficiently similar to allow generalisation across the industry (Cooper *et al.*,1984a;Rowswell *et al.*,1984;WRC,1989a). These effluents are broadly characterised by high levels of salinity, organic load, sulphide content and alkalinity (see table 2.1).

An evaluation of four individual effluent streams produced by tanneries, together with Curing Store Brine, was undertaken to determine their potential as growth media for the culture of *D.salina*.

1. Combined Process Effluent is the amalgamation of all the tanning process streams fed to a common sewer (the general practice in most tanneries), and collected at the outfall before any form of treatment has been applied. This is an average of alkaline and acid treatments and washings, which can vary between 320-744L water per hide processed (WRC,1989a).
2. Saline Hide Soak Liquor is the wash that results from the rehydration of salt cured hides at the start of the tanning process, and would be collected before its discharge to the common sewer. This accounts, on average, for between 15%-20% water utilized in a tannery (WRC,1989b).
3. Ponded Effluent refers to the contents of the solar evaporation ponds which contain pre-settled, final combined process effluent, mixed and diluted with pre-existing contents and partly treated by the facultative processes that operate in this environment (Rowswell *et al.*,1984). Given environmental exposure and different operating systems, this can be a very variable medium.

4. Curing Store Effluent is the near saturated brine solution that results from the salt curing of freshly slaughtered "green hides" and amounts to about 8L per hide treated (Cooper *et al.*,1984b).

While the salinity of certain of these effluents may vary to well below that required for the culture of *Dunaliella*, it was accepted that the proposed process could be operated in conjunction with a solar evaporation ponding treatment system, from which concentrated salt solutions could be drawn to adjust influent salinity across the required range.

3.2. RESEARCH OBJECTIVES.

To determine the basic validity of the research hypothesis by answering the questions:

1. Can *D.salina* be successfully cultivated in tannery effluent and evaporation ponds liquors;
2. Will this treatment in any way affect the subsequent production of the metabolite of interest - β-carotene?

3.3. MATERIALS AND METHODS.

3.3.1. *Dunaliella* Culture.

Dunaliella salina (Teodoresco) var *bardawil* was acquired from the Culture Collection for Algae and Protozoa, Oban, United Kingdom (CCAP 19/30). The strain was originally isolated by Ben-Amotz and Avron from a salt pond near Bardawil Lagoon, North Sinai in 1976, and was identified by them as a high β-carotene-producing strain. The organism has been patented (US Patent # 4,199,895) and deposited with the American Type Culture Collection (ATCC 30861) as *Dunaliella bardawil*. This name is probably illegitimate (Borowitzka& Borowitzka,1988b;CCAP culture data sheet). All experimentation reported in this study involved the use of pure cultures or effluent adapted cultures of this strain and unless otherwise indicated, all references to *D. salina* pertain to *D.salina* var *bardawil*.

3.3.2. Culture Medium.

The defined medium reported by Ben-Amotz and Avron (1983) for the culture of *D.salina* var *bardawil* was used in this study and arbitrarily named artificial sea water medium (ASW) to distinguish it from the re-inforced sea water media used in the early stages of algal cultivation in this laboratory.

The medium was made up with the appropriate volume distilled water, adjusted to pH 7.8 and filter sterilized through 0.45μ cellulose acetate membranes (Millipore).

NaCl M	1.5	H_3BO_4 μM	185
$NaHCO_3$ mM	50	$MnCl_2$ μM	7
KNO_3 mM	5	EDTA μM	6
$MgSO_4$ mM	5	$FeCl_2$ μM	1.5
$CaCl_2$ mM	0.3	$ZnCl_2$ μM	0.8
KH_2PO_4 mM	0.2	$CoCl_2$ μM	0.02
		$CuCl_2$ μM	0.2

3.3.3. Effluent Formulated Media.

Growth media formulated using effluents were prepared by making up to the indicated dilution of effluent using ASW, where it is described as enriched, or by the addition of tap water where it is not so described. Effluent was filtered through GF/A filters (Whatman). In all cases the final salinity was adjusted to 1.5M with NaCl (stock salt). In certain identified cases the effluent was first autoclaved (121°C, 15 bar, 20 minutes). All other formulations made use of unsterilized raw effluent.

3.3.4. Nitrogen-deficient Media.

The effects of growth in effluent, on the induction and production of β-carotene in *D.salina*, was determined by transferring cells to nitrogen-deficient medium. Nitrogen-stress has been identified as an appropriate means of inducing β-carotene production in *D.salina* (Semenenko&Abdullaev,1980;Ben-Amotz&Avron,1983,1989a). Cells were separated from the particular test growth medium by centrifugation at 2000g for 10 minutes at 15°C and washed twice in ASW medium (nitrate omitted). Centrifuged cells were resuspended in the same medium and after appropriate incubation, β-carotene was extracted as described below.

3.3.5. Growth Studies.

Media was dispensed in cotton wool stoppered 500mL conical flasks and after inoculation, incubated in a constant environment chamber - temperature 22°C, 16:8 hour light:dark cycle. Flasks were incubated on a light bench with light intensity 165μmol.m^{-2}.sec^{-1}. Each experimental result reflects a triplicate mean. Oswald (1988a) has described the use of a preliminary flask test to determine the compatibility of a specific algal/effluent system. The importance of using acclimated cultures in biological organic utilization studies in environmental engineering has been discussed by Gaudy and Gaudy (1981).

3.3.6. Cell Counts.

All algal cell counts were made in an improved Neubauer haemocytometer and results reflect a triplicate mean.

3.3.7. Effluent Analysis.

Analytical procedures for the following determinations used are as described in APHA Standard Methods (1980).

Chemical Oxygen Demand (COD)
Permanganate Value (PV)
Total Dissolved solids (TDS)
Total Dissolved Inorganic Solids (TDIS)
Suspended Solids (SS)
Nitrogen as N soluble
Ammonia as NH_3
Potassium as K
Phosphate as P_2O_5
Sulphide as Na_2S
Sulphate as SO_4
Chloride as Cl_2

Sodium, calcium, iron and chromium were determined using a Varian atomic absorption spectrometer. Certain analyses of nitrate, nitrite, ammonia and phosphate were undertaken using a Technicon Autoanalyzer II. Amino acids were assayed by the ninhydrin test following the method of Rosen (1957).

Although the validity of COD values in high salinity media are open to question due to the oxidation of chlorides by chromate, Gocke and Hoppe (1977) have found that comparisons are possible for brackish-water environments, where there is not a great deal of variation in the composition of the medium. The permanganate value (PV) is used to obtain a qualitative statement on the organic content of water (Marquart,1990). Since this method is subject to less distortion by salinity, the PV was also measured, and comparison with COD values largely confirms the observation by Gocke and Hoppe (1977).

3.3.8. Determination of β-carotene and Chlorophyll *a*.

Cells were separated from growth medium by filtration through 2.5cm GF/A filter discs (Whatman). β-carotene was extracted into 80% aqueous acetone, refiltered through 25mm 0.2μm nylon filters (LIDA) and quantified against a standard curve by measurement of absorption at 450nm. β-carotene standard (Sigma) was prepared in 80% acetone. In all cases analytical procedures were performed under conditions of low light. Chlorophyll *a* was determined according to Arnon(1949).

3.4. RESULTS.

3.4.1. Combined Process Effluent.

Growth of *D.salina* in Combined Process Effluent was evaluated in flask studies over a range of dilutions (25%, 50% and 100% = undiluted) made up with ASW medium. The Combined Process Effluent used for media formulation in this study was sourced from the Western Tanning Company, Wellington, and analysis results are reported in table 3.1.

Table 3.1 Analysis of the Combined Process Effluent from WTC, Wellington.

pH	7.2	Phosphate $mg.L^{-1}$	9.6
Conductivity $mS.m^{-1}$	1383	Potassium $mg.L^{-1}$	201
COD $mg.L^{-1}$	6240	Sodium $mg.L^{-1}$	2178
PV $mg.L^{-1}$	1463	Chloride $mg.L^{-1}$	4176
TDS $mg.L^{-1}$	8200	Sulphate $mg.L^{-1}$	312
TDIS $mg.L^{-1}$	5300	Sulphide $mg.L^{-1}$	771
SS $mg.L^{-1}$	230	Calcium $mg.L^{-1}$	226
N Soluble $mg.L^{-1}$	637	Magnesium $mg.L^{-1}$	53
Ammonia $mg.L^{-1}$	721	Iron $mg.L^{-1}$	21
		Chrome $mg.L^{-1}$	9

No algal growth was observed in Combined Process Effluent over the dilution range examined and cell lysis occurred within 24 hours in all flasks.

While this combined effluent was the average result of the various process effluents, it is possible that the analysis could vary quite widely depending on the time of day, or of the week, that individual process streams were let down. To rule out the possibility that an unrepresentative sample may partly explain the failure of the experiment, an averaged analysis was determined. Daily samples were drawn and combined to give a weekly average. Analysis results covering three weeks of operation are reported in table 3.2.

Table 3.2 Averaged analysis of Combined Process Effluent from WTC, Wellington.

	WEEK 1	WEEK 2	WEEK 3	AVERAGE
pH	7.3	7.3	7.3	7.3
Conductivity $mS.m^{-1}$	2480	2280	2540	2433
COD $mg.L^{-1}$	2205	2080	1664	1983
PV $mg.L^{-1}$	1276	767	852	965
TDS $mg.L^{-1}$	15760	14920	16430	15703
TDIS $mg.L^{-1}$	12210	11880	13270	12453
SS $mg.L^{-1}$	185	105	150	147
N Soluble $mg.L^{-1}$	671	743	728	714
Potassium $mg.L^{-1}$	73	68	69	70
Phosphate $mg.L^{-1}$	<1	<1	<1	<1
Chloride $mg.L^{-1}$	6560	6028	6737	6442
Sulphate $mg.L^{-1}$	1790	2596	2968	2451
Sulphide $mg.L^{-1}$	1365	866	819	1017

Growth studies of *D.salina* set up in the three week averaged sample of Combined Process Effluent also failed. At the time the averaged effluent samples were analyzed, the full significance of ammonia toxicity (Azov&Goldman,1982) had not yet been taken into account and it was, therefore, not separately analyzed. Nevertheless, a comparison of the two analyses is possible, and shows similarities in organic load (as PV) and nitrogen content. The ammonia concentration present in the first trial was about 8.5 times higher than the reported upper toxicity limit for *D.salina* of 5mM (Ben-Amotz&Avron,1989a).

Further evaluation of the Combined Process Effluent for culturing *Dunaliella* was not undertaken, but the role of organic load reduction and the accumulation of ammonia was identified as requiring further study.

From these results it seemed apparent that, in order to overcome the toxic effects recorded here, the effluent should be drawn at a point either upstream (i.e. at the Hide Soak stage) before the chemical degradative, ammonia producing stages are reached, or downstream where the combined effluent is mixed and diluted with partially treated ponded effluent. The subsequent evaluations of tannery effluents thus focused on Hide Soaks Liquor and Ponded Effluent.

3.4.2. Hide Soak Liquor.

Soak liquors produced in the washing of salt cured hides are fairly consistent and the sample used in this study was sourced from African Hides Company, Port Elizabeth, and the analysis results are recorded in table 3.3.

Table 3.3 Analysis of saline Hide Soak Liquor from African Hides Co., Port Elizabeth.

pH	7.2	Ammonia $mg.L^{-1}$	170
Conductivity $mS.m^{-1}$	6303	Phosphate $mg.L^{-1}$	12
COD $mg.L^{-1}$	9950	Sodium $mg.L^{-1}$	32000
PV $mg.L^{-1}$	1150	Chloride $mg.L^{-1}$	30885
TDS $mg.L^{-1}$	57100	Sulphate $mg.L^{-1}$	1559
TDIS $mg.L^{-1}$	48240	Sulphide $mg.L^{-1}$	5
SS $mg.L^{-1}$	4752	Chromium $mg.L^{-1}$	1
N Soluble $mg.L^{-1}$	143		

Dilutions for flask studies of *D.salina* growth in hide soak liquor (25%, 50%, 75%, 80%, 85%, 90%, 95% and 100% = undiluted) were made up in ASW medium. Growth curves are reported in figure 3.1 (80% to 95% results not shown).

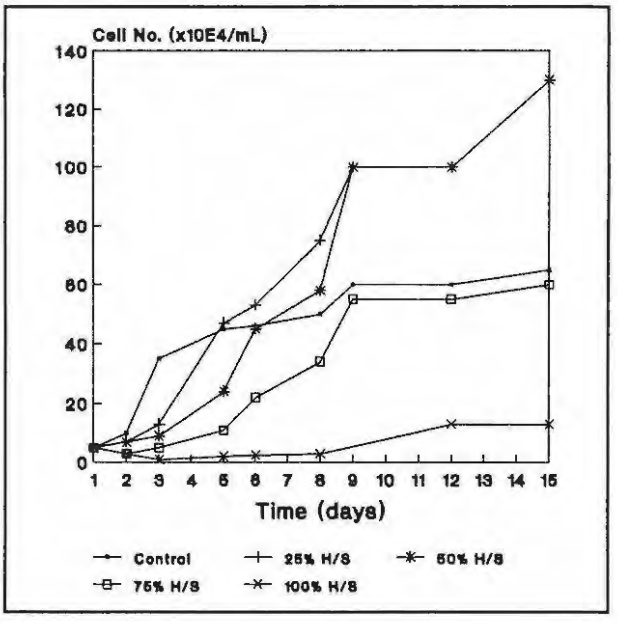


Figure 3.1 Growth of *D.salina* in various concentrations of Hide Soak Liquor. The effluent was made up with ASW medium, and compared to an ASW medium control.

D.salina inoculated in the 50% and 25% Hide Soak Liquor produced a surprising growth response, yielding twice the cell numbers of the ASW control medium. Only in those dilutions containing more than 75% effluent was the growth performance worse than the ASW control. In these dilutions, logarithmic growth commenced after an initial lag phase, and unlike the controls was sustained throughout.

To control for possible variability in effluent produced by different tanneries, growth was also measured in saline Hide Soaks sourced from three other tanneries - LIRI Experimental Tannery (Rhodes University), Exotan (Port Elizabeth) and Western Tanning Co. (Wellington). The analysis and growth study results for the LIRI Hide Soak were typical for other effluents and are reported below in table 3.4 and figure 3

Table 3.4 Analysis of saline Hide Soak Liquor sourced from LIRI Experimental Tannery.

pH	6.0
Conductivity $mS.m^{-1}$	6370
COD $mg.L^{-1}$	3203
PV $mg.L^{-1}$	439
N Soluble $mg.L^{-1}$	221
Phosphate $mg.L^{-1}$	<1

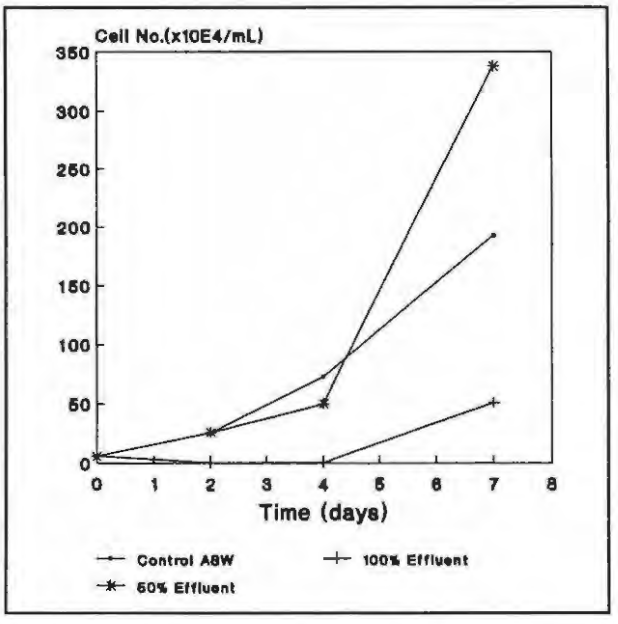


Figure 3.2 *D.salina* growth in undiluted and 50% dilution of LIRI Hide Soak Liquor. The effluent was made up with ASW medium and compared to an ASW medium control.

The growth of *D.salina* in the 50%-75% range of Hide Soak Liquor was comparable for all sources of the effluent. Algal growth in dilutions of 50% and below produced a substantial enhancement of cell growth compared to the ASW control medium. The pattern of an initial period of adaptation, followed by sustained rapid log growth, was consistent for all the effluents evaluated. While ammonia was not separately analysed for the LIRI Hide Soak, the soluble nitrogen values indicate the comparability of analyses in tables 3.3 and 3.4.

It can be noted that dilutions of roughly 50% would reduce ammonia levels to within the 90mg.L^{-1} toxicity threshold.

A comparison of algal growth in enriched and unenriched effluent was undertaken to evaluate nutrient availability and completeness of the effluent as a growth medium. Dilutions of effluent were prepared by the addition of either ASW medium (enriched) or tap water (unenriched). Salinities were adjusted to 1.5M NaCl (growth optimum) in each case. No substantial differences in growth performance were apparent between the media with the exception of the 25% effluent dilution where the growth performance of the algae in the unenriched medium was better than the enriched. The growth results are recorded in table 3.5 and indicated that Hide Soak Liquor contains complete nutritional requirements and provides an adequate alternative source of medium for *D.salina* cultivation.

Table 3.5 A comparison of *D.salina* growth in dilutions of Hide Soak Liquor. The effluent was made up with ASW medium (enriched) and tap water (unenriched).

	Cell Number ($x10^4.mL^{-1}$)	
H/S Dilutions	Enriched	Unenriched
25%	130	161
50%	237	194
75%	165	134
100%	8	24

If a period of adaptation is allowed, algal growth will eventually commence in the previously inhibitory concentrations of Hide Soak Effluent. Although there was an initial fall in numbers after inoculation, cell growth can be observed to resume after 3-4 days. This effect was demonstrated by inoculating an undiluted (=100%) and a 50% dilution of Hide Soak Liquor with cultures that had been allowed to adapt in various concentrations of the effluent for 14 days previously to the experiment (figures 3.3,3.4). Note that the lag period was reduced from about four to two days in adapted cultures.

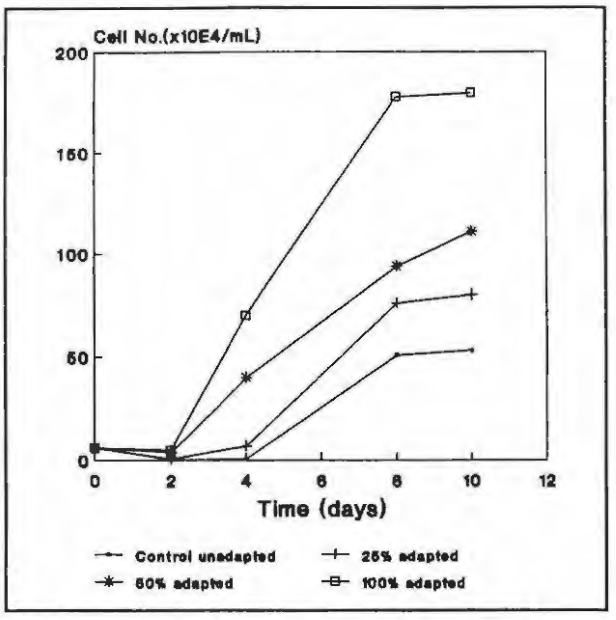


Figure 3.3 Growth of *D.salina* cells in undiluted (100%) Hide Soak Liquor. Cells were previously adapted to growth in 25%, 50% and 100% Hide Soak, inoculated unwashed (10%) inoculum, together with adapted heterotrophic components and compared to an unadapted control.

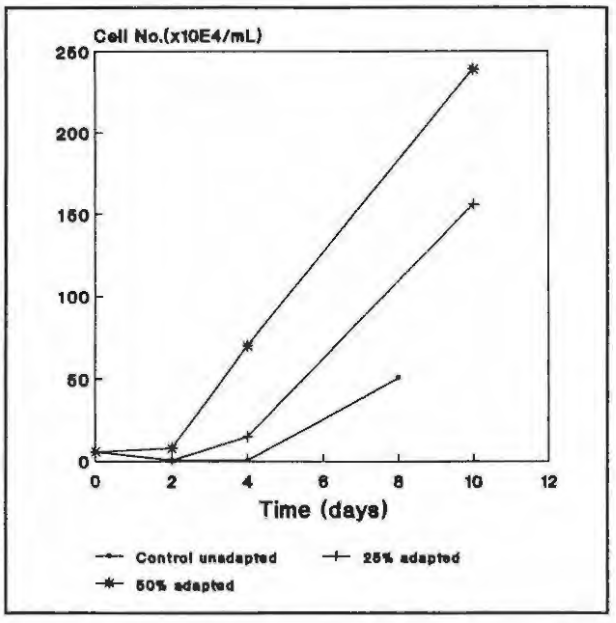


Figure 3.4 Growth of *D.salina* cells in 50% Hide Soak Liquor. The culture was otherwise treated as in figure 3.3.

The effect of growth in effluent on subsequent β-carotene production by *D.salina* was evaluated in washed cells, stressed by resuspension in nitrogen-free ASW medium. Results, recorded in figure 3.5, showed an inverse relationship between β-carotene produced per cell and the strength of the effluent dilution in which the cells were grown before their transfer to the nitrogen-free stress medium. It is apparent from these results that the suppression of β-carotene production is a concentration-related effect. Given that cells were adequately washed

it implies the carry over of some intracellularly located suppressing factor. The reversal of the effect with time is consistent with the gradual consumption or inactivation of this factor.

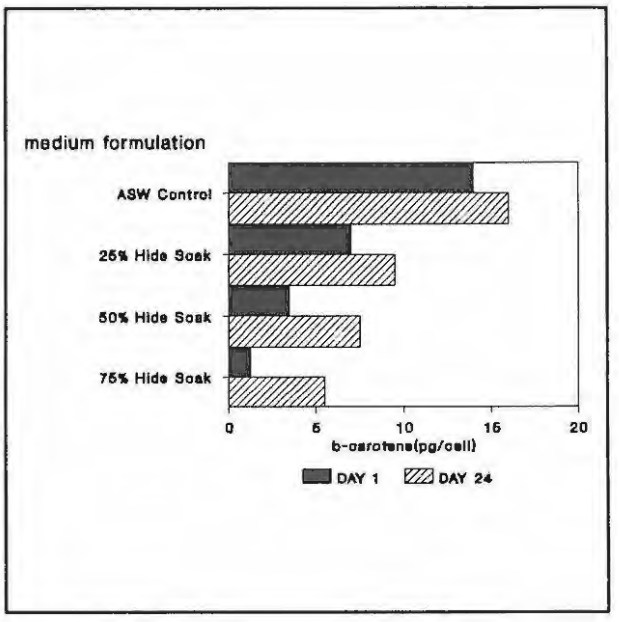


Figure 3.5 β-carotene production in *D.salina* cultures derived from growth in various dilutions of Hide Soak Liquor and control ASW medium. Cells were washed and re-suspended in nitrogen-free ASW medium and sampled at days 1 and 24. β-carotene was measured as $pg.cell^{-1}$.

Where β-carotene was measured as a function of the total culture volume (i.e. per mL medium) in the same experiment, it was found that by day 24, production in effluent-grown cultures was similar to ASW controls, with the exception of the 75% concentration (figure 3.6). This can be simply explained by a relative increase in cell numbers in those cultures which had been derived from growth in the effluent medium, the growth increase in the nitrogen-free ASW medium being in direct relationship to the strength of effluent in which the cells had been previously cultured (figure 3.7).

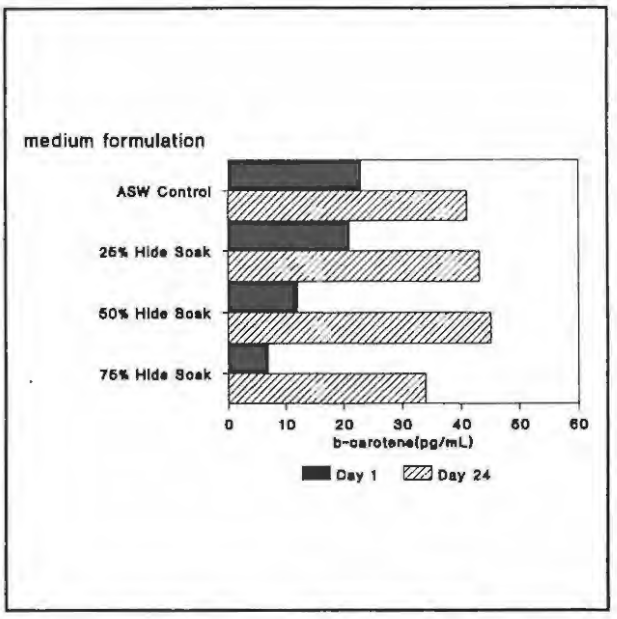


Figure 3.6 β-carotene production measured per mL medium with conditions otherwise as recorded in figure 3.5.

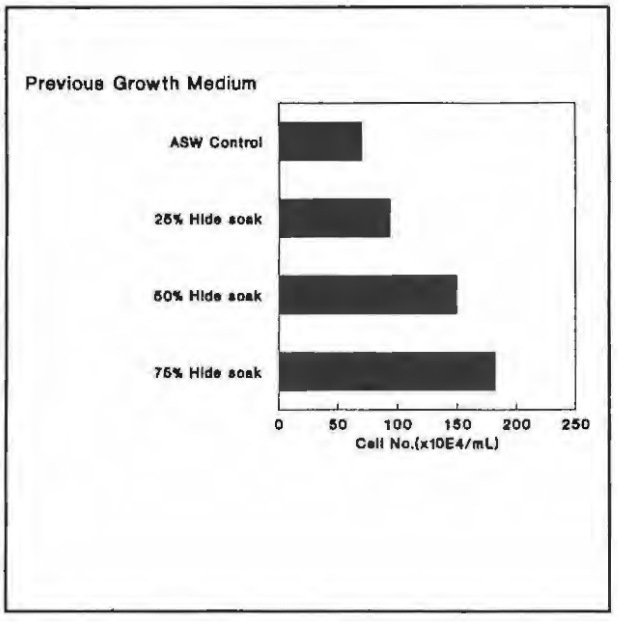


Figure 3.7 Cell counts of D.salina from cultures treated as recorded in figure 3.5.

A nutritional basis was considered for the above results which showed the unexpected continuation of cell growth, in nitrogen-free medium, by effluent-grown cultures. The nitrate, nitrite, ammonia and phosphate contents of the nitrogen-free ASW medium were determined after the resuspension of washed cells from effluent-grown and ASW-grown cultures of *D.salina*. Cells were removed from the medium by filtration after 1 and 24 days. Results are recorded in figure 3.8. While the analysis of nitrite, phosphate and ammonia showed no substantial differences (results not shown), it was found that nitrate was present in a substantially higher

amount in the medium containing effluent-grown cells compared to the ASW-grown controls. The amounts of nitrate, presumably released by the cells, showed a direct relationship to the strength of the effluent in which the cells were previously cultured. The reduction in the amount of nitrate released over the 24 day period indicated consumption and a likely explanation for the additional cell growth.

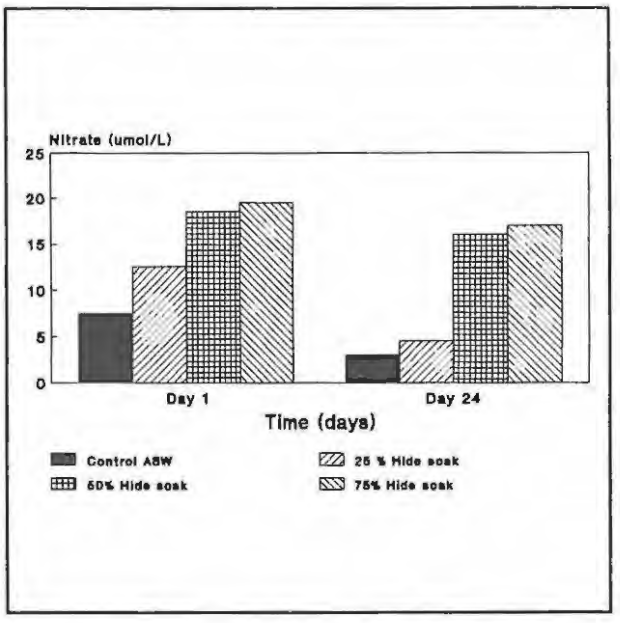


Figure 3.8 Nitrate concentration present in nitrogen-free ASW medium after the resuspension of washed cells from control and Hide Soak Liquor grown cultures. Measurement was made at days 1 and 24.

3.4.3. Ponded Effluent.

The Ponded Effluent was sourced from evaporation ponds at East Cape Tanneries (Uitenhage). The operation of this particular system has been reported in some detail by Rowswell *et al.* (1984). Analysis of the sample used in this study is reported in table 3.6.

The results of growth studies of *D.salina* in Ponded Effluent is recorded in figure 3.9. Optimum productivity was observed in the 20% effluent concentration. Cell yield in the 20% dilution at 14 days was 20% higher than in the ASW medium controls. Virtually no growth was recorded in effluent concentrations above 40%.

To account for the possible variability to be expected in Ponded Effluents drawn from different tanneries, growth of *D.salina* was also measured in Ponded Effluent sourced from WTC (Wellington), pond No.8. Results (not shown) conform to the above pattern.

The β-carotene content of Ponded Effluent-grown cells is similar to the ASW controls before subjecting the cultures to nitrogen-stress conditions: ASW control = 0.53pg.cell^{-1} and 25% Ponded Effluent = 1.47pg.cell^{-1}. However, unlike the previous results recording a suppressed β-carotene production in Hide Soaks, the yield of β-carotene in washed Ponded Effluent-grown cells, transferred to nitrogen-free stress medium, was very similar

to the ASW-grown controls:ASW control = 35.9pg.cell^{-1} and 25% Ponded Effluent = 36.5pg.cell^{-1}. The 30% Ponded Effluent sample did, however, show less β-carotene. Results are recorded in figure 3.10.

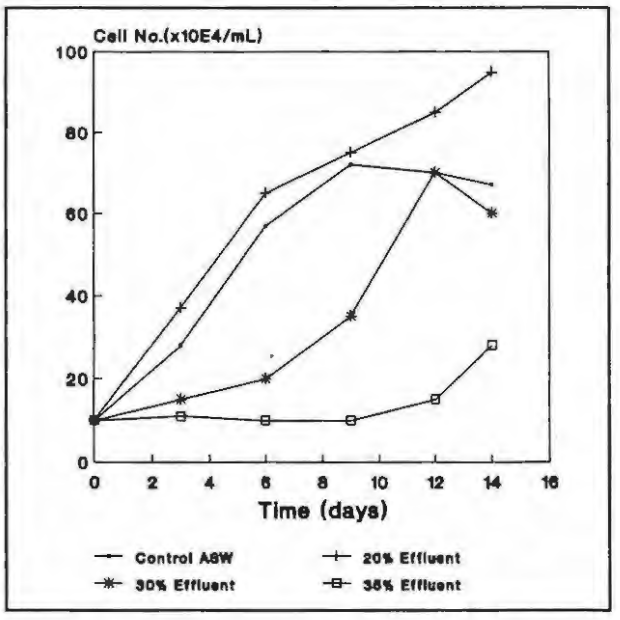


Figure 3.9 Growth of *D.salina* in various concentrations of Ponded Effluent compared to ASW-grown controls.

In the light of this result it was not surprising that the cell growth rate in the effluent-grown cells should also be higher (52% more cells) than the ASW control-grown cells after washing and transfer to nitrogen-free stress medium: ASW controls = $38x10^4$ cells.mL^{-1} and 25% Ponded Effluent-grown cells = $58x10^4$ cells.mL^{-1}. Consequently the total β-carotene yield for the culture medium (measured as pg.mL^{-1}) was also higher in the effluent-grown cultures; ASW controls = 53.8pg.mL^{-1} and 25% Ponded Effluent = 65.8 pg.mL^{-1}.

These results appear to indicate a substantially reduced, but not entirely absent, suppression effect on β-carotene production by the Ponded Effluent relative to the Hide Soak Liquor, with the carry-over of intracellular nutrients to the nitrogen-free ASW medium. Note that ammonia levels in Ponded Effluent are similar to those recorded for Hide Soak Liquor.

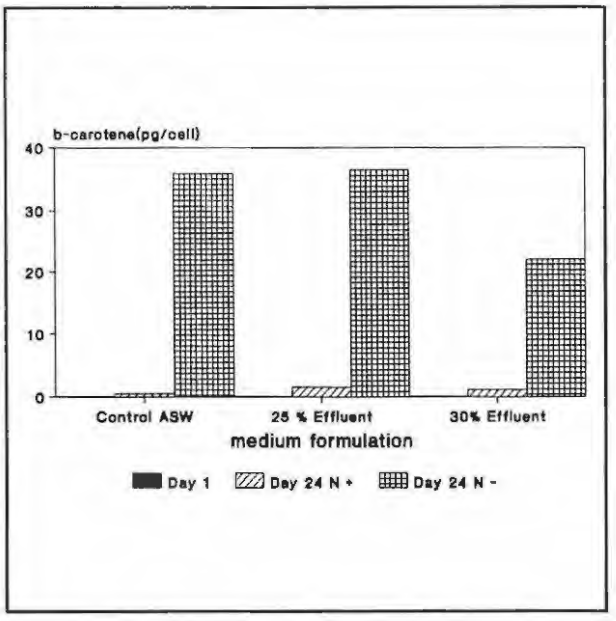


Figure 3.10 β-carotene yield per cell in washed, ASW control and Ponded Effluent-grown cells, transferred to nitrogen-free stress medium.

Table 3.6 Analysis of Ponded Effluent from East Cape Tanneries pond No.2.

pH	8.4	Potassium $mg.L^{-1}$	299
Conductivity $mS.m^{-1}$	4240	Sodium $mg.L^{-1}$	10500
COD $mg.L^{-1}$	1330	Chloride $mg.L^{-1}$	17200
PV $mg.L^{-1}$	285	Sulphate $mg.L^{-1}$	1390
TDS $mg.L^{-1}$	34100	Sulphide $mg.L^{-1}$	39
TDIS $mg.L^{-1}$	31600	Calcium $mg.L^{-1}$	32
SS $mg.L^{-1}$	112	Magnesium $mg.L^{-1}$	140
N Soluble $mg.L^{-1}$	177	Iron $mg.L^{-1}$	4.3
Ammonia $mg.L^{-1}$	163	Chromium $mg.L^{-1}$	1.4
Phosphate $mg.L^{-1}$	11		

3.4.4. Curing Store Effluent.

Effluent produced in the salt curing process for preserving green hides and skins is fairly consistent and the analysis of the sample used in this study is reported in table 3.7. The effluent was sourced from Vleissentraal Curing Store, Brakenfell.

Table 3.7 Analysis of Curing Store Effluent.

pH	6.3	N Soluble $mg.L^{-1}$	301
Conductivity $mS.m^{-1}$	81900	Potassium $mg.L^{-1}$	2480
COD $mg.L^{-1}$	7360	Phosphate $mg.L^{-1}$	<1
PV $mg.L^{-1}$	600	Chloride $mg.L^{-1}$	41700
TDS $mg.L^{-1}$	70910	Sulphate $mg.L^{-1}$	786
TDIS $mg.L^{-1}$	70060	Sulphide $mg.L^{-1}$	98
SS $mg.L^{-1}$	128		

A comparison of *D.salina* growth in undiluted, filtered and unfiltered Curing Store Brine is recorded in figure 3.11. While algal growth in this medium did not show the dramatic stimulation recorded for the previous effluents, it is apparent that clarification due to a dilution of the medium's dark colour (see figure 9.21), resulted in an improvement of light penetration and hence also cell yield.

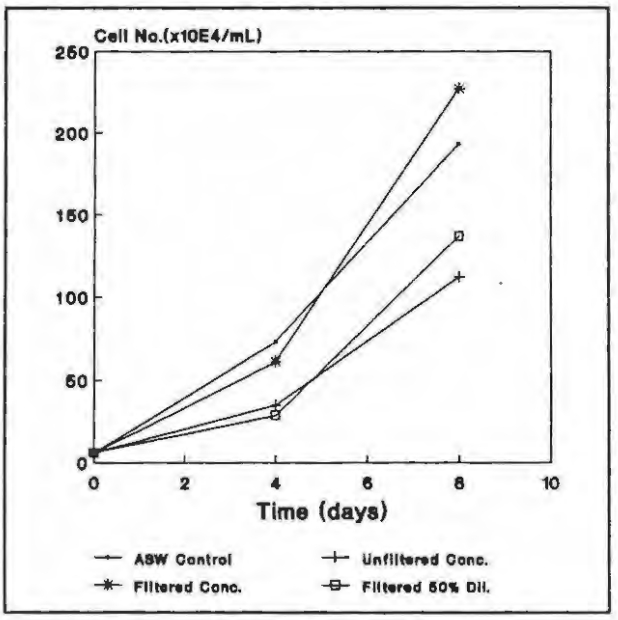


Figure 3.11 Growth of *D.salina* in concentrated and diluted, filtered and unfiltered Curing Store Effluent compared to ASW controls.

3.5. DISCUSSION.

The results of the growth studies reported above appear to provide answers to the primary questions on which the subsequent aspects of this study are to be based, and certain tentative conclusions can be drawn.

1. *D.salina* does grow in certain tannery effluents which appear to contain all the nutritional requirements necessary for effective growth. Shelef *et al.* (1980) and Oswald (1988a) have shown a similar characteristic for municipal effluents supporting algal growth. Media enrichment is unnecessary, and shows no particular growth enhancement effects. In some cases, significantly higher cell yields are produced in the effluents evaluated than in the nutritionally complete ASW medium, optimised for the growth of this alga.

2. The production of β-carotene, the metabolite of interest, was not irreversibly impaired in *D.salina* cells grown in Ponded Effluent and yield, after removal of nitrogen from the medium, was comparable to ASW-grown control cultures. The 36pg.cell^{-1} β-carotene measured in the effluent-grown, nitrogen-stressed cells, lies within the yield range reported for this organism by Ben-Amotz and Avron (1989a). Total culture yield, which takes the growth enhancement effect into account, showed a significantly higher β-carotene production potential for the Ponded Effluent cultures. Where cells were grown in Hide Soak Liquors prior to β-carotene induction in stress medium, the yield was, however, significantly reduced.

Two apparently contradictory effects appear to operate on cells grown in tannery effluent media. On the one hand full strength (undiluted) effluent can be strongly inhibitory, but on the other hand, at lower dilutions, there are pronounced growth stimulatory effects. While the cause of this effect is uncertain, a number of indications do emerge from the study. Tannery effluent, while essentially an organic medium, does contain a complex range of potentially toxic components. Besides the major process chemicals, these could include antiseptics, surfactants and various additives used in small amounts during the tanning process. The possible effects of this group of components on algal growth was identified as one of the major potential limitations of the entire concept of viable algal metabolite production in industrial effluents. Toxic components can be anticipated to be potentially present in all saline industrial effluents.

3.5.1. Growth Inhibitory Effect.

In these studies it was, nevertheless, shown that inhibition of algal growth, whatever its cause, can be overcome by either dilution, or a period of adaptation to growth in concentrated effluent, apparently making it a reversible phenomenon. The toxic effect can also be correlated with high ammonia levels in the effluent. In the Combined Process Effluent, where cells died within 24 hours, the 721mg.L^{-1} ammonia measured is some 8.5 times higher than the (90mg.L^{-1}) toxic threshold level reported by Ben-Amotz and Avron (1989a) for *D.salina,* where pH values are in excess of about 8. Where cell growth in effluent has been successful, as in the Hide Soak and Ponded Effluents, this can, in turn, be correlated with dilution factors (50% and 25% effluent) that

bring the ammonia concentration within the non-toxic range. Ammonia toxicity has been shown to be a major factor limiting algal growth in fresh water Agal High Rate Oxidation Ponds, used in the treatment of organic wastes such as sewage (Abeliovich&Azov,1976;Abeliovich,1980;Azov&Goldman,1982). Mass mortality in *D.salina* cultures have been reported by Massyuk (1966), where ammonia was released from urea used as a nitrogen source.

It seems unlikely that an excess of any major nutrient other than ammonia, can be responsible for the toxic effect on *D.salina*. Fabregas *et al.* (1986a) has demonstrated that nitrates are non-toxic up to 64mM. Phosphate levels in tannery effluents are particularly low ($<$1-15mg.L^{-1}) which is well within the optimum range (2-25mg.L^{-1}). The reported toxic threshold for phosphate is above 5g.L^{-1} (Mil'ko,1962;Borowitzka& Borowitzka,1988b). With the exception of Curing Store Brine, which contains quantities of fresh blood, potassium levels are considerably lower than the 200mM accumulated intracellularly by *Dunaliella* (Pick *et al.*,1986). Magnesium can be accumulated up to 300mM and NaCl salinity can largely be replaced with $MgSO_4$ (Fujii *et al.*,1983). *Dunaliella* can also tolerate a highly variable magnesium:calcium ratio which may change from 0.8 to 20 during the evaporation of sea water from 1M to 4M, without adversely affecting growth (Baas-Becking,1930;Borowitzka&Borowitzka,1988b). The minimum sulphate requirement is at least 2mM (Gimmler&Weiss,1987;Ben-Amotz&Avron,1989a) and tannery effluents never exceed the 30mM present in unconcentrated sea water.

While sulphate is clearly tolerated in relatively high concentrations, neither sulphide requirements nor toxicity levels are known for *D.salina*. Sulphide is used in the dehairing process and can be present in the Combined Process Effluent. It is possible that the observed adaptation effect may be partly explained by the oxidation of sulphide to sulphate when the media is incubated under aerobic conditions. Numerous attempts to demonstrate this with pre-treatment aeration failed (results not shown).

Cultures pre-adapted to effluent growth do, however, perform better than inocula grown in defined medium. There is a pronounced concentration-related effect, with the adaptation to higher strength effluent showing the best results when re-inoculated into undiluted effluent media (figures 3.3,3.4). These results seem to suggest that the effect is not due to the presence of a xenobiotic chemical added to the process stream. If ammonia is the crucial component causing the toxic effect, it is possible that adaptation may be the combined result of an avoidance mechanism acquired by the alga and the control of a bacterial population competent to deal more effectively with the proteolytic, deamination, ammonification and, possibly, deammonification functions involved in the organic degradation processes. Clearly an understanding of this toxic effect and its control will be required for a satisfactory evaluation of a *D.salina* culture system based on tannery effluent.

3.5.2. Growth Stimulation Effect.

Neilson and Larsson (1980) report that algal cultures isolated from polluted waters show enhanced metabolic capacity compared to culture collection strains. It is possible that elevated ammonia levels may also be responsible for the growth stimulation response observed. While *D.salina* is able to use ammonia, nitrate or urea as a nitrogen source (Ben-Amotz&Avron,1989a), it has been shown that ammonia stimulates growth at lower concentrations compared to nitrate (Baas-Becking,1930;Paasche,1971), although the value of this observation has been questioned and it has been suggested that this could also be due to pH effects (Borowitzka&Borowitzka,1988b). It is, however, unlikely that ammonia uptake alone, can provide a full explanation of the cell growth stimulation effects. Substantially more cell growth occurs in effluent-grown cells compared to ASW-grown controls after their being washed and transferred to a stress medium deficient in any source of nitrogen, including ammonia. In addition, β-carotene production, a very sensitive indicator of nutritional nitrogen status (Semenenko&Abdullaev,1980;Ben-Amotz&Avron,1983) is not impaired in Ponded Effluent-grown cells compared to Hide Soak-grown cells, although ammonia levels were very similar in both effluent samples.

The continuation of substantial cell production in effluent-grown cultures, after the transfer of washed cells to nitrogen-free medium, could indicate the presence of a nitrogen concentrating or storage mechanism in *D.salina*. The accumulation of an intracellular pool of nitrogen would be a controversial conclusion given the report by Ben-Amotz and Avron (1989a) that nitrogen is not stored by *D.salina* and growth ceases as soon as the (external) source of nitrogen is exhausted or removed. Flynn and Fielder (1989) however, have observed that nitrogen-replete phytoplankton may lay down excess nitrogen as storage compounds, so that subsequent low dissolved inorganic nitrogen levels do not necessarily result in nitrogen-stress. They report that these algae can utilise either the intracellular stores or external sources of nitrogen. Wikfors (1986) has found that nitrogen uptake does not necessarily denote metabolic need, and in the presence of a nitrogen abundance, the luxury uptake is stored in the form of cell protein. Fabregas *et al.* (1986b) has found excess nitrate reflected as higher levels of cellular protein in *Dunaliella*.

The direct correlation observed in these studies between effluent strength and continued cell growth (figure 3.7), and the impairment of β-carotene production (figure 3.5) and nitrate release (figure 3.8) by effluent-grown cells, adds further weight to a conclusion that a nitrogen storage mechanism is present in *D.salina*. The significance of the subsequent nitrate release by effluent-grown cells, after transfer to nitrogen-deficient medium (figure 3.8), again a concentration related effect, is uncertain.

The form in which this nitrogen is taken up and stored by *D.salina* is not known, but here again, ammonia is a potential candidate since its uptake by algae, in general, is rate dependant on its transport into the cell while nitrate uptake, on the other hand, is dependant on demand for the synthesis of the cell's nitrogenous requirements (Kerby *et al.*,1989;Kanda *et al.*,1990). The GS-GOGAT nitrogen uptake system requires less

energy for ammonia assimilation than nitrate. Ammonia is thus, generally, the preferred source of nitrogen in plants (Wetzel,1983). Excess ammonia, particularly above pH 9 in the NH_3 form, could result in its forced uptake and storage, possibly as cellular protein or some intermediate product. Here again, the lack of any impairment in β-carotene production observed in Ponded Effluent-grown cells, is not what would have been expected where the enhanced cell growth would tend to indicate the presence of an intracellular nitrogen pool. Ammonia and nitrate levels in Ponded Effluent are very similar to the Hide Soak Liquor where β-carotene inhibition did occur. This could imply that ammonia is not the sole source of the nitrogen pool.

The only significant difference between the analyses of the two effluents (tables 3.3,3.6), with respect to nitrogen content, is in organic loads as reflected in COD and PV levels. Since the organic content in tannery effluent is largely proteinaceous, it could be tentatively speculated that the source of the luxury uptake could be nitrogen in a more complex organic form such as amino acids or even peptides or proteins. Since the ponding process involves the degradation of organics, it is evident that these components would be present in far lower quantities in the Ponded Effluent than in Hide Soak Liquor. This could explain why the impairment of nitrogen stress-induced β-carotene production was not as evident in the Ponded Effluent Medium.

The proposal that an organic uptake effect is in operation, would also be directly contrary to the accepted view that *Dunaliella* is an obligate photoautotroph which is unable to utilise any organic compounds (Borowitzka&Borowitzka,1988b;Ben-Amotz&Avron,1989a). However, Antia *et al.* (1991) have reviewed reports of widespread use of dissolved organic nitrogen by phytoplankton, and quote reports of pinocytosis observed in electron micrographs of *D.primolecta* and *D.tertiolecta*, which were linked to the uptake of large organic molecules. In the latter case, the uptake of proteins such as lectins, peroxidase and ferritin through a process of receptor linked endocytosis was observed. Antia *et al.* (1991) have speculated that the accumulation of these markers in the lysosomal vacuoles would strongly suggest a nutritional significance for this process.

While the case for the involvement of an organic source of nitrogen in both the storage and growth stimulatory responses is circumstantial at this stage, the evidence would indicate that the subject requires further attention. This acquires additional importance for the development of an effluent-based *D.salina* production process, especially where β-carotene is the product of interest, and an inability to overcome its inhibition by accumulated nitrogen could invalidate the entire concept.

CHAPTER 4

THE SALINE HIGH RATE ALGAL OXIDATION POND

Summary.

An organic load reduction effect was found to accompany the growth of *D.salina* in certain tannery effluents. Results were found to be broadly comparable to literature values for fresh water HROP systems and the potential of a *Dunaliella* based saline HROP, as a process for the treatment of organic saline wastes, was identified. An indication of a mechanism for algal control of bacterial growth and hence ammonia toxicity was found. Further evidence is provided for a possible uptake of organics by *D.salina*.

4.1. INTRODUCTION.

The concept of linking the production of useful algal biomass the treatment of organic effluents had been recognised from the earliest developments in algal biotechnology (Burlew,1953). Throughout the development of the HROP system, based initially on the treatment of sewage wastes, parallel programmes are reported where the separation and application of the algal biomass produced has been investigated in animal feeding studies (Abeliovich,1986). The poor control and lack of predictability of algal species occurring and hence the low value of the algal product produced has been widely recognised (Benemann *et al.*,1980; Richmond,1986c;Oswald,1988a). These problems are characteristic of the fresh water systems that have, until now, been the subject of investigation. As previously mentioned, Soeder (1986) has drawn attention to the wider potential of the HROP as a multipurpose system for algal biotechnology production which has, hitherto, not been exploited beyond the production of low value albazod from sewage and animal feedlot wastes.

It is against this background, having established that *D.salina* can be successfully cultivated in certain organic saline effluents produced by tanneries, that the following questions arise: do commensurate organic load reduction effects accompany this growth, and is the growth sufficient to consider the HROP model linking the functions of effluent treatment and the yield of an algal product with a higher value than albazod? The demonstration of a novel saline application of the HROP concept as a mechanism for *D.salina* culture, forms the crux of the test of practicality that requires demonstration before the study can proceed with an assessment of the consequent biological problems associated with this linkage.

The results reported below was undertaken to demonstrate an organic load reduction effect in a simulated, *D.salina*-based HROP. A scale-up from flask studies would provide preliminary rate data that could allow a tentative comparison with reported literature values for comparable fresh water HROP systems. In addition, the

potential problem of ammonia toxicity, which had been identified above, needed to be further quantified. This could provide an indication for its regulation, by the manipulation of the loading rates and retention times of the system.

4.2. RESEARCH OBJECTIVES.

1. To demonstrate the simultaneous growth of *D.salina* and the reduction of organic load in saline tannery effluent;
2. To determine the rate characteristics of this process in flask studies and its scale-up to 5L photobioreactor, batch and fed batch cultures;
3. To determine whether ammonia levels could be controlled by manipulation of the feed rate.

4.3. MATERIALS AND METHODS.

4.3.1. Flask Studies.

Hide Soak Liquor (sourced from LIRI Experimental Tannery) and Curing Store Brine (sourced from Vleissentraal, Brakenfell) were clarified by aluminium sulphate flocculation cross-flow microfiltration, and by the dilution of the raw effluent with tap water. Dosage rates for the aluminium sulphate flocculation of effluents were determined using the Jar Test according to Cox (1969). Actual effluent treatment was performed in 200L drums. The later clarification procedures are described in detail in Chapter 9.

Media were made up using these clarified effluents and untreated effluent controls, salinity adjusted to 1.5M NaCl, dispensed in 100mL aliquots into cotton wool stoppered 500mL conical flasks and inoculated with a Hide Soak-adapted culture of *D.salina* to give a starting cell count of $6x10^4$ cells.mL^{-1}. Controls were set up in ASW medium (formulated as previously described).

Triplicates of each test were set up and incubated on a light bench ($165\mu mol.m.^{-2}sec^{-1}$) in a controlled environment chamber with a 16:8 hour light:dark cycle and temperature regulated at 30°C. Dark controls, measuring non-photosynthetic organic load reduction activity, were set up for each test of Hide Soak medium in foil covered conical flasks, and otherwise incubated under identical conditions. The results reflect a triplicate mean.

Flasks were incubated for 14 days and then the effluent growth media were centrifuged at 3000g for 10 minutes to remove algae. Analyses were performed as previously described. Amino acids were assayed by ninhydrin test (Rosen,1957).

Cell counts were made in an improved Neubauer haemocytometer and the recorded counts reflect a triplicate mean. Chlorophyll was measured according to the method of Arnon (1949).

4.3.2. Photobioreactor Studies.

Scale-up evaluations of flask studies were conducted in a 5L New Brunswick Bioflow III, microprocessor controlled fermentation system. The photobioreactor adaptation was effected by a bank of four 50cm, cool white fluorescent tubes arranged around half the vessel diameter and providing constant illumination. The use of adapted fermentation vessels in algal modelling studies has been described by Markl (1980). Dissolved oxygen (DO), temperature and pH were logged at 15 minute intervals. Temperature was controlled at 30°C and agitation set at 100rpm. The Ingold DO electrode was calibrated by sparging uninoculated medium with nitrogen and oxygen as described by the manufacturers. Headspace pressure was equilibrated with atmospheric pressure via a condenser vent, which also provided the only source of possible gas exchange between the internal and external environments.

Hide Soak Liquor was sourced from LIRI Experimental Tannery, clarified by filtration through GF/A filters (Whatman), diluted to 25% with tap water and salinity adjusted to 1.5M NaCl. It was accepted that the high organic content of the medium would inactivate any residual chlorine in the tap water.

1. Batch Cultures.

In the batch studies an inoculum of effluent-adapted *D.salina* culture was added and the digestion process allowed to proceed for 9 days. Samples were drawn daily via the sampling device and analyses carried out using methods previously described.

2. Fed Batch Cultures.

In the fed batch studies the same procedures, as reported above, were used for setting up and inoculating the photobioreactor. At intervals, a percentage of the reactor volume was removed and replaced by an equivalent amount of fresh, filtered 25% Hide Soak Liquor. Feed rates evaluated were 2%, 5%, 8% and 10%. Feed addition was made daily with the exception of the 8% and 10% rates where the culture recovery times were longer than 24 hours. The recovery time was determined as the time required, after feed addition, for the DO to return to the level prevailing before that addition. This reflects the difference between the totals of oxygen production and consumption during this time.

The oxygen uptake rate was calculated from the slope of the DO utilization curve after the addition of feed as described by Gaudy and Gaudy (1981). Gocke and Hoppe (1977) have described the calculation of the carbon oxidation rate based on oxygen utilization values and an assumed respiratory quotient value of 0.8. The oxygen production rate was determined by degassing the steady state system for each dilution rate, with nitrogen gas connected to the reactor sparging device. Degassing was discontinued when DO reached zero and its recovery, due to photosynthetic reaeration, was plotted as DO against time. The conversion of % DO to μmoles oxygen.L^{-1} was based on the extrapolation of oxygen saturation in 1.5M salinity sea water at STP from tables provided by Grasshof *et al.* (1983).

4.4. RESULTS.

4.4.1. Flask Study - Hide Soak Liquor.

Organic load reduction and *D.salina* growth was evaluated in both unclarified and clarified Hide Soak Liquor and the contribution of the photosynthetic component to the biological oxidation process was evaluated by comparison with dark incubated controls. As could be anticipated, the algal inoculum in the dark incubated flasks disappeared within a few days, with the death and lysis of cells observed microscopically. This provided an effective non-photosynthetic heterotrophic control for the algal effect on the biological oxidation process.

Figure 4.1 shows an inverse correlation between medium clarity and algal growth, with lower cell numbers in the less clear media. That this was a light effect, and not the result of either the dilution or the removal of toxic components by the clarification treatments, was demonstrated by the higher cellular levels of chlorophyll *a* in the algae grown in the more turbid media. An increase in chlorophyll production in response to reduced light availability has been demonstrated for *D.salina* by Ben-Amotz (1987).

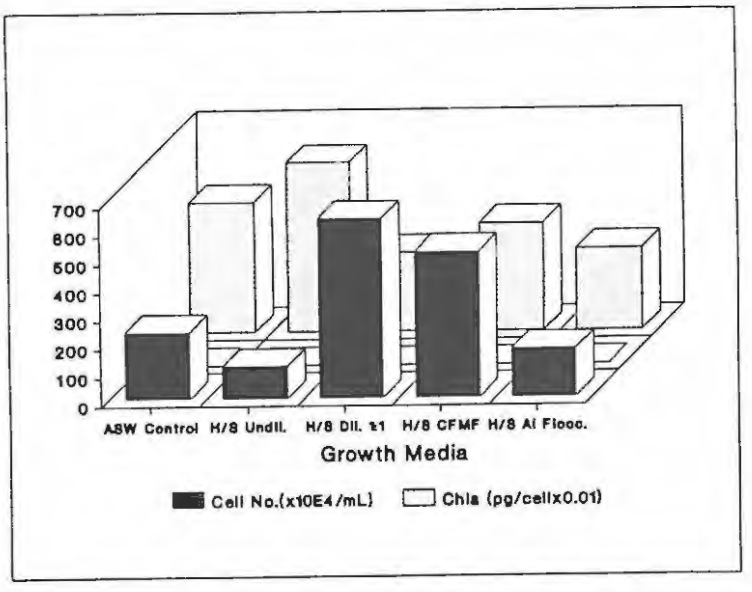


Figure 4.1 *D.salina* growth in unclarified and clarified Hide Soak Liquor. The medium was clarified by dilution, cross-flow microfiltration and aluminium sulphate flocculation. Growth measured as cell number and chlorophyll *a* content in pg.cell^{-1}, and compared to ASW-grown controls.

Analytical results for the various media formulations used are reported in table 4.1 and the relevant organic load reduction data are arranged graphically in figures 4.2, 4.3, 4.4 and 4.5.

Table 4.1 A comparison of the biological oxidation of Hide Soak Liquors, clarified by various processes, and incubated in the presence (light) and absence (dark) of *D.salina* growth.

	Hide Soak Undilute			Hide Soak Dil 1:1		
	Before	After		Before	After	
		Light	Dark		Light	Dark
pH	7.8	8.3	8.2	7.7	8.2	8.2
Conductivity mS.m^{-1}	9490			9390		
COD mg.L^{-1}	3203	574	1638	1601	642	406
PV mg.L^{-1}	439	93	111	219	95	40
SS mg.L^{-1}	70			35		
TKN mg.L^{-1}		137	539		60	84
Ammonia mg.L^{-1}		96	172		17	81
Nitrate mg.L^{-1}	<1	<1	<1	<1	<1	<1
Phosphate mg.L^{-1}	<1	<1	<1	<1	<1	<1
Potassium mg.L^{-1}	120			88		
Sulphide mg.L^{-1}	<1			<1		
Chloride mg.L^{-1}	5390			5180		
	Aluminium Floc.			CFMF Clarification		
	Before	After		Before	After	
		Light	Dark		Light	Dark
pH	7.6	8.1	8.1	7.6	8.1	8.2
Conductivity mS.m^{-1}	9460			9620		
COD mg.L^{-1}	2205	500	420	915	474	310
PV mg.L^{-1}	413	65	64	102	51	39
SS mg.L^{-1}	45			35		
TKN mg.L^{-1}		81	130		25	63
Ammonia mg.L^{-1}		53	132		4.3	64
Nitrate mg.L^{-1}	<1	<1	<1	<1	<1	<1
Phosphate mg.L^{-1}	<1	<1	<1	<1	<1	<1
Potassium mg.L^{-1}	135			111		
Sulphide mg.L^{-1}	<1			<1		
Chloride mg.L^{-1}	5250			5500		

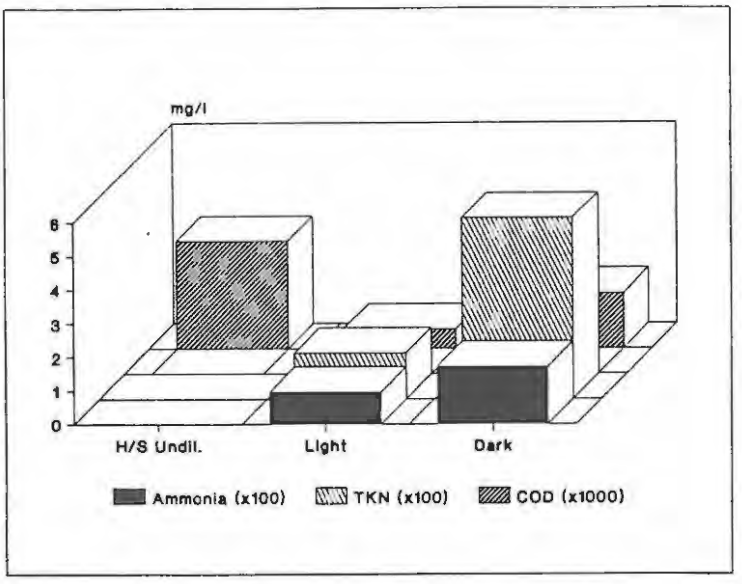


Figure 4.2 Organic load reduction in unclarified Hide Soak Liquor (undiluted) comparing biological oxidation in the presence (light) and absence (dark) of *D.salina* growth.

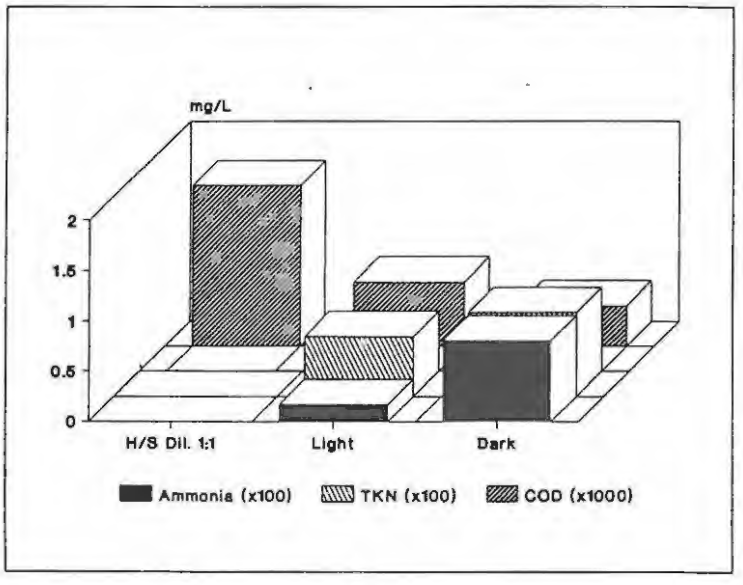


Figure 4.3 Organic load reduction in diluted Hide Soak Liquor (partially clarified by dilution 1:1 with tap water) comparing biological oxidation in the presence (light) and absence (dark) of *D.salina* growth.

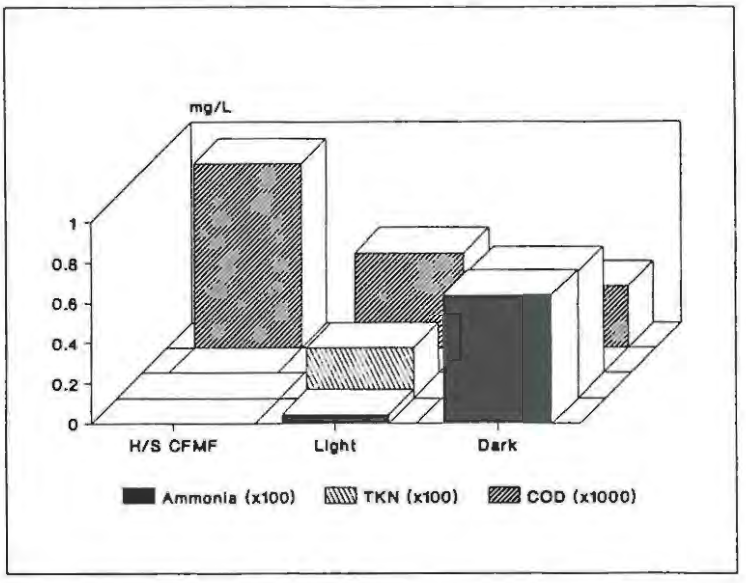


Figure 4.4 Organic load reduction in CFMF Hide Soak Liquor (clarified by cross flow microfiltration) comparing biological oxidation in the presence (light) and absence (dark) of *D.salina* growth.

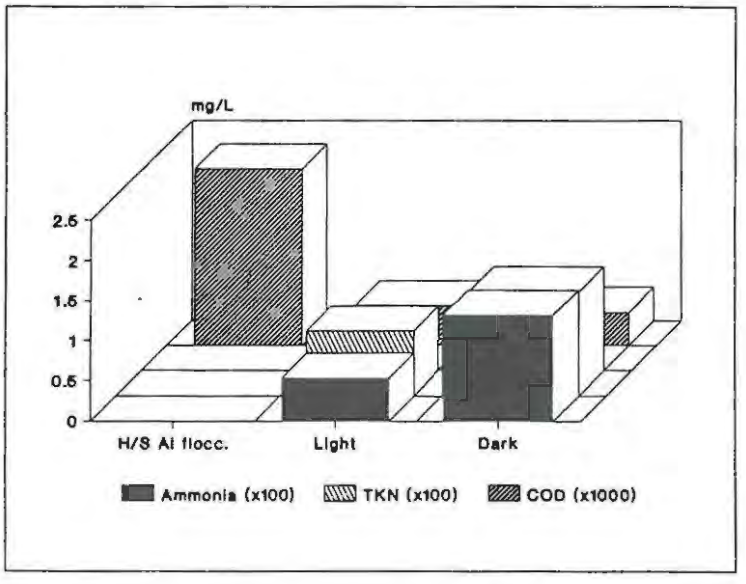


Figure 4.5 Organic load reduction in aluminium treated Hide Soak Liquor (clarified by aluminium sulphate flocculation) comparing biological oxidation in the presence (light) and absence (dark) of *D.salina* growth.

The results showed a substantial organic load reduction effect (COD reduced between 50%-80%) for both incubations in the presence and absence of *D.salina* growth.

A general trend is apparent which seems to show that lower algal cell numbers (figure 4.2) correlate with a significantly more effective reduction of COD when compared to dark incubated heterotrophic controls (an 82% reduction compared to 49%). However, where algal cell growth was higher, as found in the more clarified effluents, (figures 4.3,4.4,4.5) the COD reduction, while still substantial was no longer better than the dark treatment. Arrangement in order of increasing clarity of medium approximates increasing cell number where the difference in efficiency is 4% for aluminium flocculation, 15% for diluted effluent and 18% for the CFMF treatment. This result is further illustrated in figure 4.6 which shows that the larger percentages of COD remaining in the media can be associated with the presence of higher algal cell numbers.

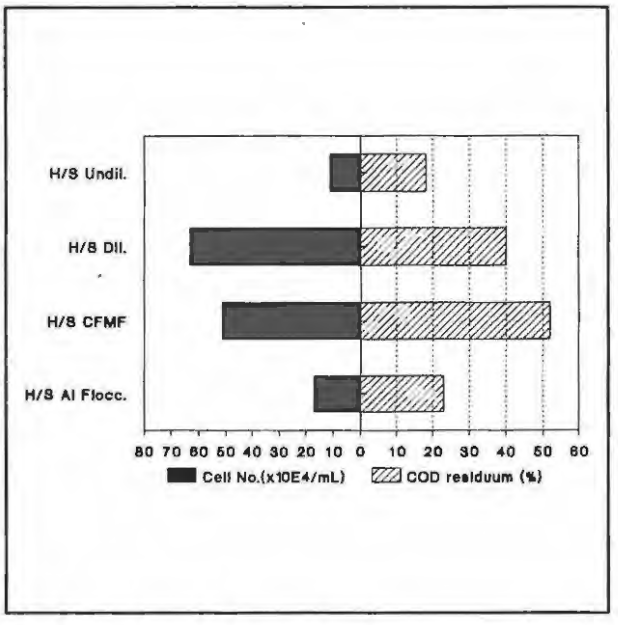


Figure 4.6 A comparison of *D.salina* cell number and the percentage of the original COD load remaining in the treated Hide Soak Liquor.

While starting levels of total nitrogen (TKN) and ammonia present in the medium were not recorded, it is evident from the final results in all cases, that algal growth correlates with the substantial of these components as is evident in the comparison of light and dark treatments. In this case, where algal cell numbers were higher (i.e. in clarified effluents) a greater part of the ammonia fraction of the total nitrogen was shown to be removed from the medium (figures 4.3,4.4,4.5). A larger residuum of ammonia remains in the medium where algal cell numbers were lower (figure 4.2) or entirely absent (dark treatments figures 4.3,4.4,4.5). The direct correlation of percentage ammonia uptake with cell number is further illustrated in figure 4.7.

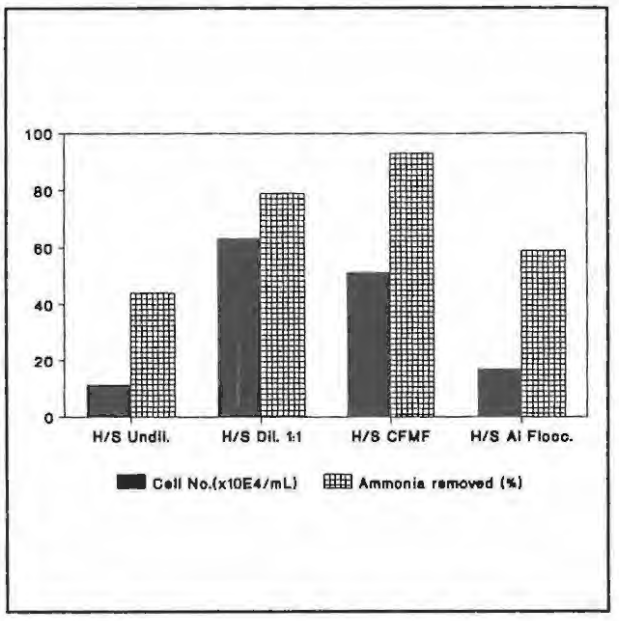


Figure 4.7 A comparison of cell number and percentage ammonia uptake attributable to algal growth by comparing light and dark treatments.

Since pH remains fairly constant for both light and dark treatments, it is most likely that the ammonia is removed by algal growth rather than by physical stripping effects. In the various dark heterotrophic treatment, TKN is almost completely converted to ammonia in all except the unclarified effluent, which may be accounted for by the higher initial strength (COD) of this medium. The presence of algae therefore appears to exert some effect retarding the heterotrophic conversion of protein to ammonia. The ability of algae to control the rate of the bacterial degradative process may account, in part at least, for the adaptation effect of the algae to effluent growth conditions noted in the previous chapter.

Nitrates and phosphates remain constant at levels below $1 mg.L^{-1}$ for both light and dark treatments. This indicates that ammonia is not oxidised further to nitrate and its absence in the dark treatment serves to confirm that a rapid, unmeasured uptake of nitrate by the algae has not taken place.

4.4.2. Flask Study - Curing Store Brine.

Flask studies, similar to those reported above, were undertaken demonstrating the organic load reduction effects in Curing Store Brine, of an algal biological oxidation treatment with *D.salina*. Results are recorded in table 4.2.

Although algal photosynthetic (light) and heterotrophic (dark) treatments were not compared in this study it was, nevertheless, evident that a substantial reduction in organic load, similar in extent to the Hide Soak study, had taken place over the 14 days of the experiment. Here again, high algal cell numbers correlate with the

clarification of the medium, and an elevated cellular chlorophyll *a* content in algae grown in the unclarified media, indicates that this was primarily a light effect.

Table 4.2 Analysis of biological oxidation of Curing Store Brine in the presence of *D.salina* growth. The analysis of unclarified effluent was compared to CFMF clarification and CFMF clarification plus 1:1 dilution, both before and after algal growth.

	Unclar. Eff.		CFMF Clar.		CFMF Dil1:1	
	Before	After	Before	After	Before	After
pH	6.7	8.7	6.9	8.7	6.9	9.1
Conductivity $mS.m^{-1}$	11720		6700		4700	
COD $mg.L^{-1}$	7904	1860	3245	1026	1622	574
PV $mg.L^{-1}$	1084	410	234	114	117	69
TKN $mg.L^{-1}$		88		56		36
Ammonia $mg.L^{-1}$		85		55		19
Nitrate $mg.L^{-1}$	<1	<1	<1	<1	<1	<1
Phosphate $mg.L^{-1}$	<1	<1	<1	<1	<1	<1

4.4.3. Photobioreactor Study - Batch Culture.

The digestion, by algal oxidation, of 25% Hide Soak Liquor in the Bioflow III photobioreactor was examined in a 5L batch culture. The results recorded in figure 4.8 are typical of two further repetitions of this study.

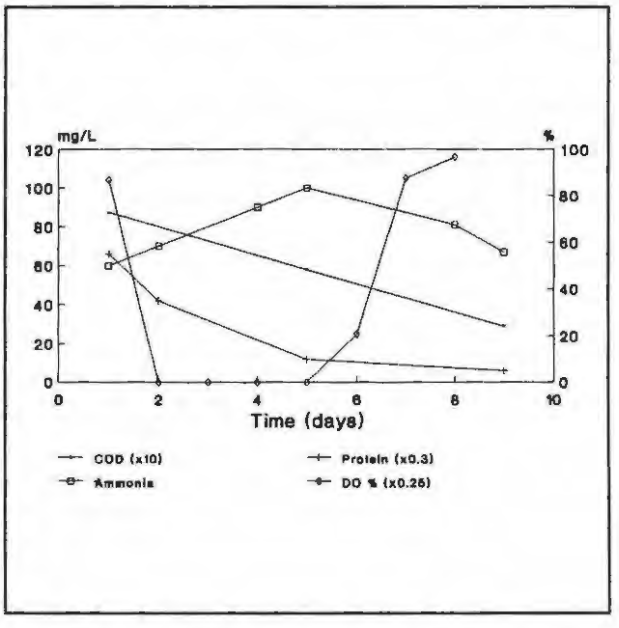


Figure 4.8 A 5L photobioreactor batch study of organic load reduction in 25% Hide Soak Liquor subjected to algal biological oxidation by *D.salina*.

The overall trends that emerged from this study appear to have been anticipated by the prior flask study. The reduction in organic load (COD) over the nine day period was 68%, with a removal rate of 7.4%.day^{-1}. The fall in COD was reflected in a parallel reduction in protein content and a rise in ammonia level, which was also observed in the flask studies. The subsequent reduction in ammonia after day 5 is not related to an accumulation of nitrate which was reduced from low initial levels to zero by day nine. Rising levels of chlorophyll *a* indicate continued algal growth throughout the treatment, again indicating algal uptake of ammonia (results not shown).

The oxygen demand is high during the initial stages of the process with measurable DO falling to 0%. Where photosynthetic O_2 production starts to exceed heterotrophic consumption, this coincides with the removal of the major part of the organic load indicating a substantial respiratory consumption in the system.

4.4.4. Photobioreactor Study - Fed Batch Culture.

The results of the algal oxidation of 25% Hide Soak Liquor in the 5L photobioreactor operated in the fed batch mode are reported in figure 4.9 and 4.10. Protein levels are given here as an indication of organic load to establish a more direct relationship with the appearance of ammonia. A close correlation between COD and protein level reduction was noted in the batch study. Proline was measured in addition to total amino acids to provide a specific indication of collagen degradation.

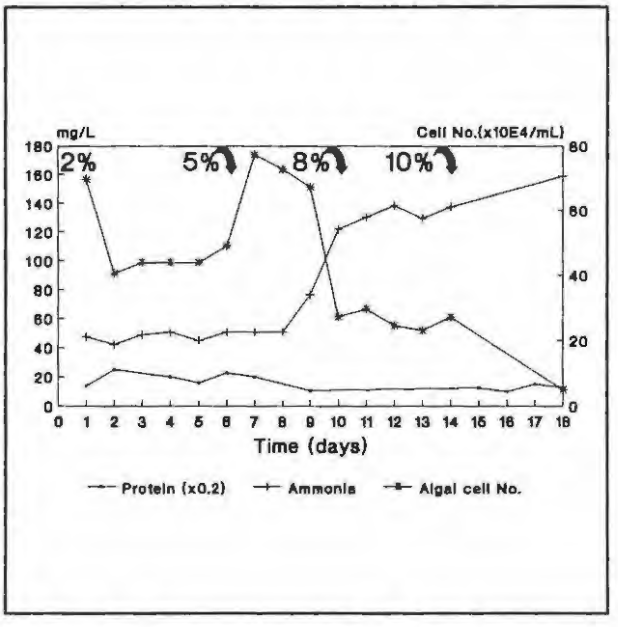


Figure 4.9 A 5L photobioreactor fed batch study of algal oxidation of 25% Hide Soak Liquor measured as *D.salina* cell number, ammonia and protein levels. Changes in feed rates from 2% to 5% to 8% and to 10% were applied as indicated by arrows.

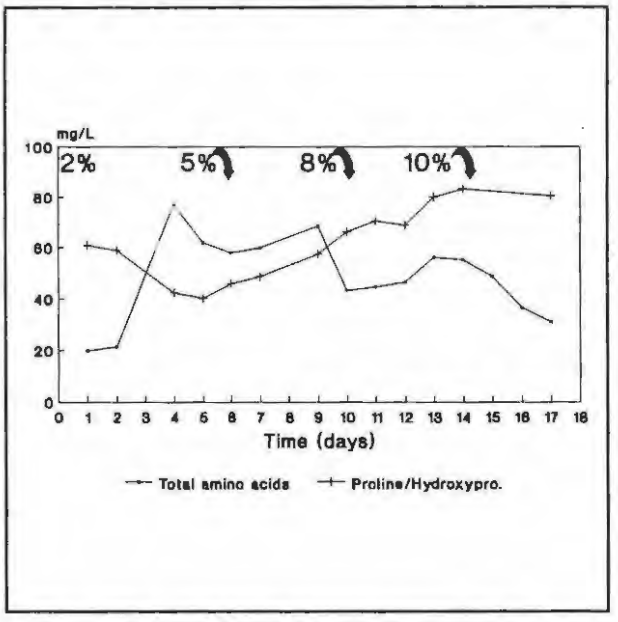


Figure 4.10 Levels of total amino acids and proline in the fed batch study reported in figure 4.9.

Each percentage effluent loading regime produced approximate steady state conditions with regard to the various parameters measured. The rate of protein degradation exceeded the loading rate even at the 10% feed addition level, and remained constant at a low value of between 2-4mg.L^{-1}. However, ammonia showed a stepwise increase that correlated with increased loading of the reactor. Algal cell numbers showed a concomitant decline as the ammonia concentration exceeded approximately 80mg.L^{-1}. No conversion of ammonia to nitrate was apparent from these results. Amino acid accumulation profiles indicated different rates of oxidation, and possibly utilization for these components of the system.

The relationship of changes in dissolved oxygen and pH to the addition of 2% feed to the reactor is recorded in figure 4.11. Only the 2% result is shown but a similar DO response was found for each subsequent stage in the fed batch process. The rates of oxygen uptake, carbon oxidation and the recovery times of the system were calculated as described in methods and are recorded in table 4.3.

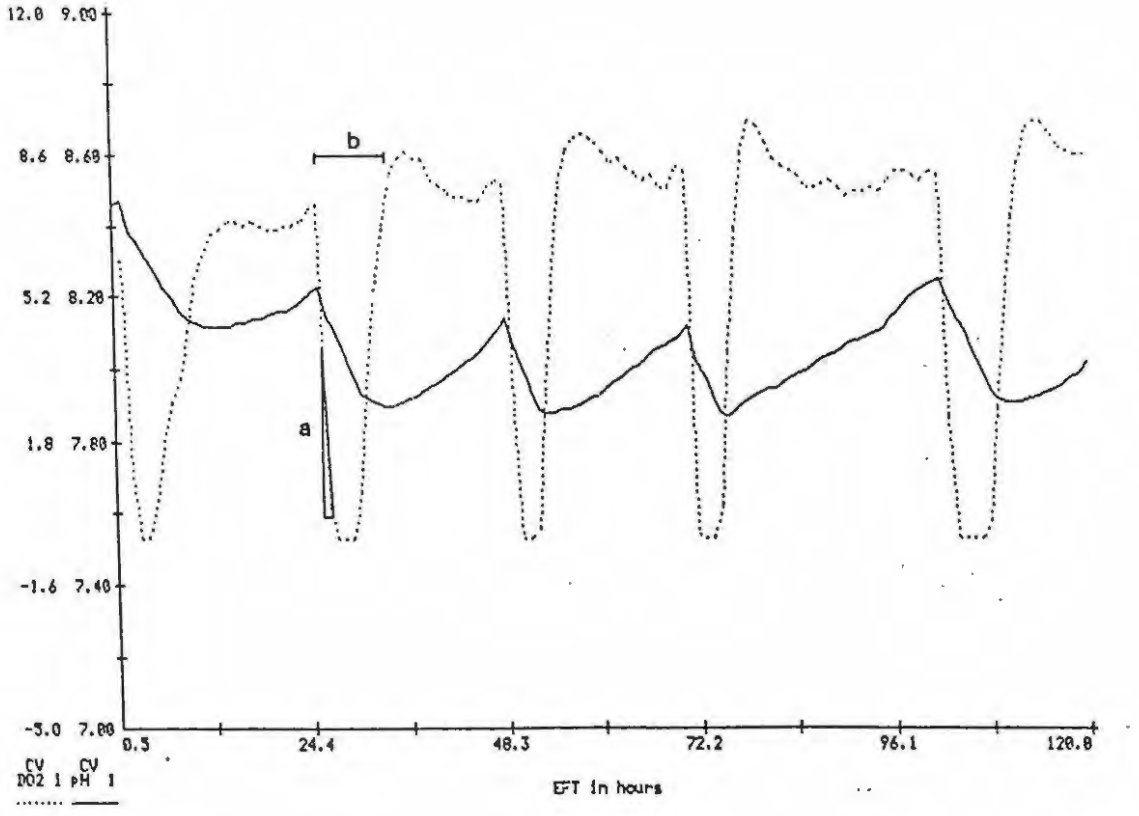


Figure 4.11 The response of DO and pH to fed batch conditions in an adapted culture of *D.salina* grown in 25% Hide Soak with a feed rate of 2%.day^{-1}. Oxygen demand is calculated from slope a and recovery time given by b.

Table 4.3 Oxygen uptake, carbon oxidation rates and the recovery times for the Hide Soak fed batch study with varying feed rates, as indicated.

Feed Addition Rate (%)	Oxygen Uptake Rate ($mg.O_2.L^{-1}.hr^{-1}$)	C Oxidation Rate ($mg.C.L^{-1}.hr^{-1}$)	Recovery Time (hours)
2	0.11	0.088	7.06
5	0.12	0.096	23.04
8	0.15	0.12	28.1
10	0.38	0.3	36.6

The oxygen demand of the system increased with each increase in the rate of organic loading. The recovery rate records the return of DO to steady state levels existing prior to a particular addition of feed. This reflects the time when, primarily heterotrophic, respiratory oxygen consumption exceeds photosynthetic production by the

algae (table 4.3). An indication is provided of the time within which it could be expected that a repeat of feed to the system would be accepted without the threat of overload. While the recovery time remains fairly constant within steady state conditions for each level of feed addition, it showed an increase in direct relationship with every increase in rate of feed addition. Daily feed addition rates of between 5% and 8% appeared appropriate to allow recovery of the system within a 24 hour period. Where this recovery period is exceeded, at 8% and 10% feed additions, ammonia levels rose sharply and the culture failed.

The algal oxygen production rate was determined by degassing with a nitrogen purge, during the post recovery steady state phase, for each level of feed addition. The results were substantially similar for all rates, and those recorded at the end of the 8% feed addition phase are reported in figure 4.12.

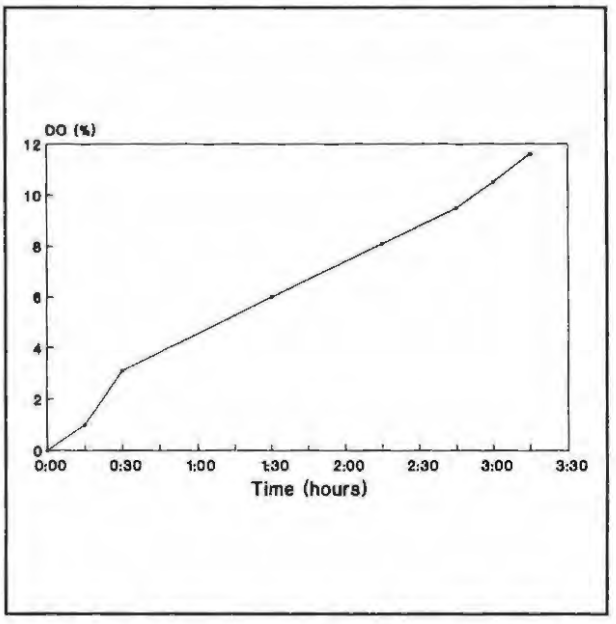


Figure 4.12 Photosynthetic oxygen production rate in a batch culture fed 8% Hide Soak Liquor.day^{-1}. Measurement was made during photosynthetic re-aeration of a nitrogen degassed culture while in steady state.

A reaeration rate of 2.67%.hour^{-1} was derived from the slope of the plot of DO against time. The oxygen production rate was calculated as a function of algal biomass: 6.13μmol O_2.mg chla^{-1}.hour^{-1} (= 0.0532mg O_2.mg chla^{-1}.hour^{-1}).

This calculation assumes no substantial biomass change during the short period of measurement required for this type of calculation (Pirt, 1975), and reflects total photosynthetic oxygen production less oxygen consumption. Oxygen consumption provides an indication of aggregate respiratory function as noted by Gaudy and Gaudy (1981) for oxygen consumption measurements in sewage sludge systems. The fixation of carbon by the algae in the fed batch system can be calculated from oxygen production during steady state conditions. Again heterotrophic consumption at this stage must be considered to be small. The fixation rate was calculated at 0.53mg C fixed.mL^{-1} oxygen evolved (according to Lind, 1974).

4.5. DISCUSSION.

The organic load reductions effected by the algal oxidation process are broadly consistent for the flask and the batch and fed batch studies. The effects are also similar for the two different effluent media formulations evaluated. The result may allow the generalisation that, where *D.salina* growth is recorded in tannery effluent, it is accompanied by a reduction in organic load due to algal/bacterial activity. This reduction is characterized by the rapid, almost complete degradation of the protein content of the effluent, with the production of amino acids and ammonia among the other products of the process.

4.5.1. The Effect of Ammonia.

A significant uptake of ammonia by the algae has been observed and in certain cases serves as the sole source of inorganic nitrogen. Ammonia is also not measurably further oxidized to nitrate in this system, and literature reports seem to endorse the finding that this absence of nitrate is not due to its immediate removal by algal utilization. Skerry and Parker (1985) have recorded the suppression of nitrification and denitrification by algal growth in oxidation lagoons, and Post (1977) also records the absence of nitrates and nitrites from the Great Salt Lakes, Utah. De Pauw and De Leenheer (1977) report the preferential uptake of ammonia by algae which is related to a lower energy expenditure by the cell, compared to nitrate utilization. Goldman and Peavy (1979) have found that nitrates are only used by algae after the complete removal of ammonia. Cresswell and Syrett (1981) and Ahmed and Hellebust (1988) have demonstrated the suppression of nitrate transport in the presence of ammonia. Flynn and Fielder (1989), reports that nitrate reductase is not measured in cells growing in the presence of high concentrations of ammonia, and in the mM range the repression of the nitrate transport and assimilation process becomes complete.

The confirmation from the experimental results, of a substantial utilization of ammonia by effluent-grown *D.salina*, and the sensitivity of the HROP system to ammonia toxicity, lends weight to a provisional conclusion that this component could be partly responsible for the growth and the toxicity effects that have been observed and reported above.

Flynn and Fielder (1989) report a rise in α-amino nitrogen, and a concomitant decrease in ammonia levels for algal cultures grown in the presence of excess ammonia as the sole nitrogen source. Sjoblad *et al.* (1979) have studied chemotaxis in *Dunaliella* and record attraction by ammonia and certain amino acids, but not nitrate. They have proposed that this may reflect both a preference and an uptake efficiency for these components. The above findings, however, do not in themselves explain any growth stimulation effects. In fact, Goldman and Peavy (1979) have found that different sources of nitrogen do not affect the growth rates of marine algae. A role for ammonia in the growth stimulation effect will need further elucidation.

The toxic effects of ammonia in fresh water HROPs has already been noted (Abeliovich&Azov,1976). Ammonia is a potent uncoupler of photosynthesis and Abeliovich (1986) reports that when it exceeds 2mM, at pH 8.1-8.2,

photosynthesis is inhibited and the system must rely on respiration for ammonia removal. This occurs at a 5-6 times slower rate. Algal photosynthesis is thus effectively under ammonia control in the HROP system. As the pH exceeds 9, the NH_3 form predominates and this, according to Chevalier and de la Noue (1985), penetrates the cell by membrane diffusion rather than active transport, and is thus toxic at very low levels.

Results from the fed batch study indicate that the accurate determination of loading rates will be crucial to regulating ammonia toxicity in the HROP. Additional factors that have been suggested for controlling toxicity are the manipulation of pH and the extension of retention times (Abeliovich,1980). Shelef *et al.* (1980) record that up to 27% of nitrogen loaded can be lost by ammonia stripping in open ponds and Koopman *et al.*,(1980) note that this is increased by mixing and elevating pH and temperature.

4.5.2. Heterotrophic Activity.

Where DO recovery time in the fed batch studies exceeds 24 hours (ie. between 5% and 8% daily loading rates), ammonia steadily accumulated, resulting finally, in the collapse of the algal component of the system. However, at lower loading rates the steady state achieved for ammonia levels may be due, not only to algal uptake, but also to a control of the rate of deamination, exercised possibly by the algae themselves. The incomplete conversion of TKN to ammonia in the presence of algae, compared to the dark heterotrophic system (figures 4.3,4.4,4.5) and the accumulation of amino acids during the fed batch, indicates an algal effect on the bacterial deamination process. Bacterial plate count numbers did not rise above $6x10^5$ which is surprising in this highly organic medium. Counts in fresh water HROPs do not rise above $1x10^6$ according to Abeliovich (1986).

In contrast to the findings of Gocke and Hoppe (1977), who have demonstrated a direct relationship between numbers of saprophytic bacteria and BOD levels in the eutrophic waters of the Kiel Fjord, Dor (1980) and Dor and Svi (1980) have reported low bacterial numbers in algal oxidation ponds treating organic wastes. They have concluded that the algae exercise an inhibitory control over bacterial numbers. This would serve to control excessive deamination and hence the production of toxic ammonia. It is also of interest to note that as the feed rate increased from 2% to 10%, bacterial numbers remained largely unchanged at $\mu = D$ (where μ is the specific growth rate and D is the dilution rate). This, however, is not a conclusive demonstration of an inhibition of bacterial growth. Pirt (1975) has noted for quasi-steady state microbial systems, continuously or repeatedly fed, that the organism automatically adjusts its specific growth rate so that it remains equal to the dilution rate. While this condition is likely to break down at the beginning of a feed cycle, it has been shown that bacteria can immediately adjust to a shift up in μ of about 20%. The algal:bacterial ratio remained roughly constant throughout the fed batch experiment.

4.5.3. Organic Uptake.

Another possible explanation for the reduced ammonia levels found to coincide with algal growth in the light-incubated, effluent digestion flask study, is that the algae may be removing TKN in the organic form. Abeliovich and Weissmann (1978) have found that 50% of carbon assimilated by algae in the fresh water HROPs they studied, takes place by direct algal heterotrophic uptake. They consider the small numbers of bacteria present to be insufficient to account for the full mineralization effects that are observed in HROP. *Scenedesmus* has been shown by these authors to utilize at least four amino acids - glycine, arginine, leucine and phenylalanine.

The question that arises is whether *D.salina* conforms to this observation and whether it could account for the growth stimulation and nitrogen storage effects already noted. A difference was observed in the fed batch between the accumulation of proline and the steady removal of total amino acids. The amino acids proline and glycine are components of collagen, the major protein expected to be present in hide-derived effluents. This selective amino acid utilization could be due to either *D.salina* or bacterial activity. Clearly this is an area requiring further clarification, not only to satisfy a need for theoretical insights, but also for the practical reason of determining that part of the COD and protein levels loaded to the system which could be expected to lead to ammonia toxicity.

4.5.4. A Saline HROP.

The results of the batch and fed batch systems can be used to compare the potential of the saline HROP noted here with fresh water HROPs previously recorded in the literature. Borowitzka and Borowitzka (1989) have commented on the value of scale-up studies in designing large-scale algal operations. It must, nevertheless, be pointed out that the experimental results reported here were obtained under the artificially controlled conditions of a closed environment, constant illumination and temperature control at 30°C, which imposes limitations on direct comparisons with large scale processes. The criticism of saline COD values by Gocke and Hoppe (1977) must also be borne in mind when making comparisons with fresh water HROP's. Benemann *et al.* (1980) have reported COD removal in a 0.25ha experimental pond, treating municipal sewage effluent. The results are largely comparable to those recorded above for the batch study - a 55% COD removal at 7 days compared to a 67% COD removal at the 9 day retention times observed here. An algal density of 3.8mg.L^{-1} was reported which is approximately twice the value measured in these batch studies.

Abeliovich (1986) assumes average values for municipal sewage (BOD = 600-700mg.L^{-1}), of which approximately 60% is soluble. He records a 90-99% removal of the soluble BOD fraction with a four day retention time in sewage HROPs. If a similar ratio of soluble to insoluble organics is applied to the Hide Soak Effluent batch study, a 90% COD removal is accomplished at the 9 day retention time. Again, given that chlorophyll levels in this study were approximately half the level encountered in Benemann's HROP, which could account for the longer retention time, the results are roughly comparable.

Assuming that Abeliovich's figures of up to 50% heterotrophic consumption of organics by HROP algae also applies to the *Dunaliella* HROP, the rough comparability of the two systems indicated by this study, provides a further suggestion of the possible uptake of organic nitrogen by *D.salina*. Comparisons of laboratory studies are necessarily tentative, and would need to be confirmed in further scaled-up outdoor ponding trials. Since this was a technical study designed to demonstrate initial feasibility, further scale-up was not undertaken here. The results do, however, provide an indication that a saline HROP based on the halophile *D.salina* could be a viable option for use in the combined function of removing organics from saline effluents, and at the same time, producing an algal monoculture with the potential of high value metabolite yields.

PART B:

FUNDAMENTAL CONSIDERATIONS

It has been established that *D.salina* not only grows in tannery effluent, but in certain specific effluents it grows very well, with a growth stimulation effect producing higher cell yields than complete, semi-defined media. These effluents appear to provide complete nutritional requirements for the growth of the alga. A reduction in organic and inorganic nutrient loading occurs during algal growth, and offers the potential advantage of an effluent treatment operating simultaneously with the production of algal cells and metabolites. The growth studies reported appear to establish a satisfactory basis on which the further development of the research hypothesis can be founded.

A number of fundamental questions have been identified to which answers will be required in order to demonstrate the operational feasibility of the idea. Two problem areas in particular have emerged from the preliminary study - the toxicity of the medium to cells under certain conditions and the repression of β-carotene production in *D.salina* grown in the presence of high levels of nutritional nitrogen.

1. TOXICITY.

The toxicity problem will need to be addressed in practice, by a process design that will prevent the cells being overwhelmed by ammonia accumulation during the breakdown of the proteinaceous waste. The rate of ammonia production must in some way be linked to its consumption by algal cells or its removal by stripping effects. A potential model for integrating cell production with an effluent treatment function exists in the algal HROP process, developed by Oswald and others, for the treatment of fresh water organic wastes. Experimental results in this study allow a provisional conclusion that a saline system, based on the growth of halophilic algae and treating saline organic effluents, can be expected to work in a similar fashion to it's fresh water equivalent. The manipulation of feed rates and residence times has been used successfully to regulate ammonia levels (Abeliovich,1980) and experience gained in the operation of the fed batch would tend to support that observation. While this investigation was conducted at 1.5M NaCl, in a closed environment, it is known that predation and competition would need to be controlled by elevating salinities to about 2.5M NaCl if predominantly *D.salina* cultures were to be maintained (Ben-Amotz&Avron,1989a).

Experimental results also seem to indicate that the algae are able to exercise some form of control over the rate of ammonia production from the proteinaceous effluent, by limiting bacterial numbers in some way. The bacterial numbers are surprisingly low and this has also been observed in fresh water HROP systems (Dor,1980;Abeliovich,1986). Although the evidence is much more circumstantial, there is some indication, at

least, that the alga may be able to remove nitrogen in the organic form, prior to the ammonification stage. This could be as amino acids, peptides or protein.

If the organic utilization effect does occur at all, it would be important to quantify the degree to which total protein loaded to the system can be expected to convert to ammonia and the amount of organic nitrogen taken up directly, if at all, in the case of *Dunaliella*. This would in turn determine the loading rate that could be tolerated by the system. Clearly an investigation of these speculations and a more precise demonstration of the effect is required.

2. NITROGEN REPRESSION.

The nitrogen repression of β-carotene production in certain effluent-grown cells, however, presents a problem that could invalidate the entire concept of producing this metabolite in a highly nitrogenous medium such as the proteinaceous tannery effluents. The high nitrogen levels in the effluent will not permit the process of simultaneous cell growth and β-carotene accumulation within the same production unit, as is the current practice in commercial, defined medium culture systems (Ben-Amotz&Avron,1989a).

The presence of high levels of nitrogen in the effluent medium is further complicated by the apparent luxury uptake and storage of nitrogen by *D.salina* grown under these conditions. Here again, it is important to determine whether organically bound nitrogen plays a role in this effect. It appears from the experimental results that the ability of the cells to induce β-carotene production is not irreversibly impaired hy growth in tannery effluent, and repression can be reversed by transfer to a nitrogen-deficient stress medium.

The need to uncouple cell growth and metabolite induction, in an effluent-based *D.salina* production system raised the idea of a multistage process. In such a design, cell growth could be optimised in an effluent based first stage, with full advantage taken of the growth stimulation and cell yield enhancement effects that have been demonstrated for the effluent. Following this, separation and transfer of cells to a nitrogen-deficient stress medium would allow more precise control of the metabolite induction process. Cell separation technology to effect this transfer could, however, present an insurmountable problem, and will be dealt with later.

A further potential problem that could arise in the stress unit is the monitoring of the metabolite induction process in cells derived from a complex organic environment, and containing, possibly, unknown quantities of accumulated stored nitrogen. The optimization of synergistic stress effects could prove complicated where several environmental factors are operating on the alga simultaneously, especially given the variability of industrial effluents. In this case it could be important to be able to monitor the induction of the stress response using a more immediate parameter than product formation, the appearance of which could be separated from the induction event by several days or even weeks in a large-scale production system. An accurate fixing of the time of induction could, in turn, determine subsequent management procedures.

The requirement for an analytical tool to enable the exercise of a more precise control over the stress induction event could be met by recent reports that stress in the algae may be mediated by hormonal control (Tietz *et al.*,1989;Hirsch *et al.*,1989;Cowan&Rose,1991). The possibility exists, in theory at least, that an understanding of the hormonal mediation of stress may provide a practical biotechnological instrument for the manipulation of metabolite production by *Dunaliella*.

3. RESEARCH OBJECTIVES.

Research priorities were identified on the basis of the above discussion. The issues of toxicity and its control, the nature of the growth stimulus, the nitrogen pool and the regulation and control of the stress induction process for β-carotene production, are fundamental questions that require answering. However, the effective demonstration of feasibility will also require the concurrent evaluation of the more practical constraints impacting on the biotechnology of the process, such as the appropriate mass cell culture and separation techniques that will be required. The fundamental considerations will require evaluation before proceeding to the issues of practical application.

4. EXPERIMENTAL PROGRAMME.

The experimental programme was designed to provide answers to certain of the fundamental considerations that have arisen including:

1. To determine whether *D.salina* does take up and utilize organic compounds from its growth medium, and if so, to determine by what mechanism this process is accomplished;
2. To determine whether *D.salina* is able to exercise a form of control over ammonia production by bacterial activity;
3. To determine whether the inhibition of β-carotene production caused by the presence of excess nitrogen in the growth medium can be overcome by:
   a. process design changes that separate the stages of production into cell growth and metabolite accumulation unit operations,
   b. a more precise control over the manipulation of the stress induction process than is available with currently used technology;
4. To investigate reports that a plant hormone may be involved in mediating stress events in the algae, and to evaluate potential for its use as an indicator or probe of the induction of β-carotene synthesis in *D.salina*.

CHAPTER 5

ORGANIC NUTRITION IN *D.SALINA*

Summary.

A role for organic compounds in the nutrition of micro-algae, previously demonstrated in phytoplankton, has been confirmed in this study for *D.salina*. Speculation on the nature of the uptake process includes the identification of a positive control mechanism whereby the algal population, in a saline HROP, effects the availability of ammonium derived from bacterial deamination of amino acids, by regulating the release of glycerol. A practical advantage in manipulating this release is identified in the potential for using the saline HROP as a disposal process for treating organic solid wastes. The presence of an inducible deaminase, active on the surface of *D.salina*, has been provisionally excluded.

5.1. INTRODUCTION.

The role of dissolved organic nitrogen in determining the primary productivity of marine and freshwater ecosystems has received considerable attention and its function in the nutrition and cell biology of phytoplankton has been recently reviewed by Flynn and Butler (1986), Kaplan *et al.* (1986), Kerby *et al.* (1989) and Antia *et al.* (1991). While some controversy surrounds the question of whether organic compounds are released by healthy micro-algal cells (Hellebust,1970,1985;Hellebust&Lewin,1977;Sharp,1977),there is a considerable body of evidence demonstrating the uptake of amino acids (Nielson&Larsson,1980;Raven,1980;Eddy,1982;Flynn& Butler,1986); peptides (Lang *et al.*,1979;Senkpiel *et al.*,1978;Storey&Wagner,1986); protein (Oliviera& Huynh,1989); purines and pyrimidines (Shah&Syrett,1984) and hypoxanthine (Oliviera&Huynh,1990).

5.1.1. Uptake of Organics.

Antia *et al.* (1991) have noted that the uptake of amino acids has become an active area of research given recent appreciation of the quantitative nutritional role of this component. Qafaiti and Stephens (1989) have shown that ^{14}C-labelled glycine is taken up by *Platymonas*, with the retention of amino nitrogen and the respired carbon skeleton released as CO_2. Flynn and Syrett (1986) report that in *Phaeodactylum tricornutum* the bulk of amino acid uptake is incorporated directly into protein without the release of any part of the carbon skeleton. The presence in *Chlorella* of distinct storage and metabolic pools has been suggested (Antia *et al.*,1991).

It has been shown that amino acid uptake by the micro-algae can inhibit the utilization of other sources of nitrogen such as nitrate (Bilboa *et al.*,1981;Ricketts,1988), urea (Kirk&Kirk,1978) and ammonium (Flynn&Wright,1986). The rate of glycine uptake has been found to be inversely related to cellular nitrogen

content and amino acid transport is induced by nitrogen deprivation (Flynn&Butler,1986). Ricketts (1988) examined glycine uptake in *Tetraselmis* and showed that the organism is able to draw simultaneously on mixed sources of nitrogen, which is the situation most likely to occur in a natural environment. *Platymonas* has been found to take up sufficient glycine to supply 10% of its nitrogen requirement (Lu&Stephens,1984).

The actual mechanism by which amino acids are internalised has been the subject of some controversy, but the permeability coefficients are generally low and uptake by passive diffusion and leakage is thought to be small (Raven,1980). Flynn and Syrett (1986) have demonstrated amino acid co-transport across the plasmalemma with sodium via a Na^+/K^+ anti-porter.

The presence of inducible surface deaminases have been recently demonstrated in certain micro-algae which effect an external deamination with the uptake of the released ammonia and the accumulation of oxo acids in the medium (Munoz-Blanco *et al.*,1990).

The above observations contradict the traditional view that saprophytic bacteria are the only users of dissolved organic nitrogen, undertaking its mineralization and releasing free ammonia, which is in turn utilized by phytoplankton in primary production. Bacteria clearly do play a role in this process and are thought to be able to outcompete phytoplankton for most organic substrates in marine ecosystems (Wheeler&Kirchman,1986). Ducklow *et al.* (1986) have noted, however, that their role is more important in the mineralization than in the assimilation of organic matter into higher trophic levels.

The comparative quantitative roles of bacteria and micro-algae in the utilization of organics is not clear. It has been argued that the products of bacterial protein hydrolysis and deamination are largely consumed by the bacteria themselves, given their relative C:N ratios which indicate a higher nitrogen requirement for bacteria (3:1) than for micro-algae (6.6:1) (Wheeler&Kirchman,1986). The process seems to be partly controlled by the C:N ratio of the matter being degraded (Billen,1984). Where C:N >10 the uptake and utilization by bacteria of ammonium produced in deamination exceeds its release to the medium.

The evidence suggests that the saturation kinetics for micro-algal uptake of organics is low in natural aquatic ecosystems (Antia *et al.*,1991). Clearly this situation does not prevail in the HROP treating organic effluent where Abeliovich (1986) has found that the algal component may be responsible for up to 50% of organics removed.

There are, however, micro-algae for which the uptake of organic nitrogen could not be demonstrated (Antia *et al.*,1991), and in some cases uptake, where present, is not linked to cell growth. The position of *D.salina* in this regard is uncertain and despite reports of glutamine utilization noted by Borowitzka and Borowitzka (1988b), Flynn and Fielder (1989) found no uptake by *D.primolecta* for 20 amino acids tested. Oliviera and

Huynh (1989,1990) have recently reported a pinocytotic uptake of hypoxanthine and protein by *D.tertiolecta*.

5.1.2. Release of Organics.

Hellebust (1974,1985), Fogg (1983), Wetzel (1983) and Antia *et al.* (1991), have reviewed the copious literature demonstrating the release by phytoplankton of photo-assimilated carbon, and the role this plays in the nutrition of marine and fresh water ecosystems. Conditions favouring this release include any conditions affecting growth such as nutrient limitation, inhibitors and the stationary growth phase. The evidence for the release of organic compounds by *Dunaliella*, however, is contradictory.

Jones and Galloway (1979) have reported high rates of glycerol release by *D.tertiolecta* and Gimmler and Moller (1981) noted a direct relationship between endogenous glycerol levels and diffusion rates in *D.parva*. Enhuber and Gimmler (1980) have measured the reflection coefficients of the *D.parva* cytoplasmic membrane and did not find an unusually low permeability. They report this organism can leak up to 30% of endogenous glycerol per day, which accounts for 4.5% of total carbon assimilation. They suggest that this organism follows a strategy of tolerance rather than avoidance with regard to minimizing glycerol loss. Huntsman (1972) has calculated that as much as 60% of photosynthate may be released per day by phytoplankton, under certain conditions. Grizeau and Navarro (1986) have proposed a commercial glycerol production process based on natural leakage from immobilized cells of *D.tertiolecta*.

In direct contrast to these reports, Sharp (1977) has argued that leakage observed in phytoplankton is the result of methodological anomalies and that healthy algal cells do not excrete organic compounds.

Ben-Amotz (1975) and Borowitzka *et al.* (1977) reported no release of glycerol at salinities above 0.6M NaCl and concluded that glycerol measured is due to release from lysed dead cells. Wegman *et al.* (1980) showed an association between temperature and glycerol release and reported minimal leakage below 35°C. Brown *et al.* (1982) undertook a comparative NMR study of glycerol permeability in pig erythrocytes, lipid vesicles and *D.salina* and concluded that the algal cytoplasmic membrane does, in fact, represent a genuine anomaly, showing exceptionally low permeability to glycerol.

While it is possible, of course, that several mechanisms may be involved and that these may alter over the time course of the stress response, cognisance, nevertheless, needs to be taken of the fact that a large measurable concentration gradient of 10^4:1 is maintained across the membrane, with respect to glycerol in *D.salina* under hypersaline conditions (Brown *et al.*,1982).

5.2. RESEARCH OBJECTIVES.

Clarity with regard to the uptake, utilization and release of organics by *D.salina* is of importance to the current investigation given the inhibition of β-carotene accumulation in organic effluent media and the potential identified for the halophilic algal HROP to serve as a process for the treatment of organic wastes.

The uptake of amino acid and protein will be examined in this chapter and mechanisms that might account for the process are dealt with in an ultrastructural study of waste-grown *D.salina* cell biology, reported in the following chapter. Both glycine, as a major component of collagen, and bovine serum albumin (BSA) can be anticipated to be present in fresh Hide Soak Liquor and, as such, have been used as reference components in this study.

The following questions were identified:

1. Is *D.salina* able to internalise amino acids and protein from its growth medium?
2. What role does bacterial deamination play in this process?
3. Is glycerol released by *D.salina* and what effect does this have on the deamination process?

5.3. MATERIALS AND METHODS.

Culture conditions, media formulation and analytical procedures, except where otherwise indicated, were undertaken as previously described. Where cultures were washed preparatory to the evaluation of nutritional nitrogen sources, cells were separated by centrifugation at 3000g for 10 minutes at 10°C and resuspended in 1.5M NaCl, and the process repeated three times.

5.3.1. Glycine Uptake.

Washed cells were resuspended in fresh ASW medium to a culture density of $30x10^4$ cells.mL^{-1} with a reduction in bacterial numbers to $< 1x10^2$, determined as colony forming units (CFU) on 1.5M NaCl salinity adjusted plate-count agar (Difco). Chloramphenicol was added to a final concentration of 50μg.mL^{-1}.

[^{14}C]-glycine (3959mBq.mmol^{-1}) (Amersham), was added to the washed culture and incubated with continuous illumination (100μmol.m^{-2}.sec^{-1}) at 30°C. At time intervals of 5 minutes, 12 and 24 hours, 1mL of culture medium was removed, centrifuged, washed a further 3 times and the cells resuspended in 100μL distilled water and transferred to aqueous scintillant (Packard). Culture supernatant and total culture (cells + medium) were likewise transferred in 100μL aliquots to aqueous scintillant and counted in a Beckman LS3150T scintillation counter and results adjusted to dpm values. The results reflect a triplicate mean.

Binding specificity was determined by the duplication of the above procedure except with the addition of 5mM unlabelled glycine (Merck) to the culture medium. The reversibility of [^{14}C]-glycine binding to the algal cell surface was assessed by removing an aliquot of culture from the label study after 5 minutes, washing 3 x as

described above, and resuspending the cells in fresh ASW medium+5mM unlabelled glycine with continuous mild agitation. After 10 minutes cells were again washed 3 x in fresh ASW medium, resuspended in 100μL distilled water, transferred to aqueous scintillant and counted. The reduction in the count was derived by subtracting this value from that acquired for the 5 minute sample described above.

The incorporation of label into protein was determined by the lysis of washed cells with distilled water containing 2% TCA, 2% SDS and 2% methanol. The extract was filtered onto GF/A discs (Whatman) and washed 3 times with 10mL TCA. Filter discs were dried and counted in non-aqueous scintillant.

5.3.2. Bovine Serum Albumin Uptake.

To 5mL *D.salina* culture ($35x10^4$ cells.mL^{-1}), 600μL [^{14}C]-BSA (140GBq.mmol.$^{-1}$) (Amersham) was added and incubated at 30°C under constant illumination (248μmol.m^{-2}.sec^{-1}). At time intervals 5 minutes (t=0), 6, 12, 24, 36 and 48 hours 500μL samples were removed, filtered on cellulose nitrate filters (Millipore) and washed 4x10mL fresh ASW medium. Filters were dried, placed in aqueous scintillant (Packard) and counted.

5.3.3. Chlorophyll Fluorescence.

A Turner model 112 Digital Filter Fluorometer was adapted for chlorophyll measurement by the installation of a blue lamp (excitation range 390-450nm), an 405nm excitation filter (Corning 5-62) and an 680nm emission filter (Corning 2-60). The instrument was fitted with a continuous flow cell (5mL light path) and connected to a New Brunswick 5L Bioflow III microprocessor controlled fermentation system operated as a photobioreactor and illuminated with cool white light. Temperature, pH and DO were measured continuously. A culture of *D.salina* was maintained at log phase, at a cell concentration of approximately $30x10^4$ cells.mL.$^{-1}$ and chlorophyll 1.1μg.mL.$^{-1}$ The photobioreactor was connected to the fluorometer by 60cm clear Tygon tubing (6.5mm ID) through a Watson Marlow 501 peristaltic pump and a return line of equal length. A flow rate of 130mL.minute^{-1} was established and the system allowed to stabilize for at least 1.5 hours before readings commenced. Fluorescence output was set by blank adjustment to a reading of approximately 50% prior to the commencement of each experiment. Change around this point was recorded as percentage relative fluorescence. Fluorescence traces were acquired on a Lloyd flatbed recorder attached to the fluorometer. The use of continuous flow fluorometry for the *in vivo* measurement of algal chlorophyll has been described by Berman (1972) and Kiefer *et al.* (1972).

5.3.4. Glycerol Release.

Washed cultures of *D.salina* were prepared as described above, to which were added 5mM glycine, 50μg.mL^{-1} chloramphenicol, both singly and in combination. Glycerol was assayed by enzyme analysis (Boehringer). Bacterial numbers were determined as CFU on 1.5M NaCl plate-count agar (Difco).

5.3.5. Heterotrophic Activity.

Heterotrophic potential was determined as the measurement of glycine utilization by a *D.salina*/bacterial co-culture derived from the 5L batch HROP described in Chapter 4 and following the method of Parson and Strickland (1962) as modified by Gocke (1977). The [^{14}C]-glycine (Amersham) was mixed in a known ratio with unlabelled glycine (Merck) producing a concentration range of 25 through to 500μg C.mL^{-1} and transferred to 500mL screw top flasks. Formalin (0.3mL) was added to one flask as a control. A vial containing 1mL ethanolamine was attached to the neck inside one flask of a duplicate, for each concentration in the range. To each flask 100mL HROP culture was added, sealed and incubated for 2 hours with mild shaking, at 30°C and constant illumination (100 μmol.m^{-2}.sec^{-1}), after which further activity was stopped by the addition of 0.3mL formalin. The CO_2 produced was released by acidification with 0.5mL 1N HCl and the ethanolamine transferred to 10mL aqueous scintillant and counted. The contents of the duplicate flask in each case was filtered through 0.25μm cellulose acetate filters (Millipore) washed 3 times with 10mL fresh ASW medium, dried and transferred to non-aqueous scintillant and counted.

5.4. RESULTS.

5.4.1. Nitrogen Utilization.

A comparison of *D.salina* growth on different sources of nitrogen is reported in figure 5.1. The results showed that the alga not only used glycine as a sole nitrogen source for cell growth, but an enhancement effect of over 50% was observed for this amino acid compared to nitrate and ammonium, and 67% where it was used in combination with nitrate.

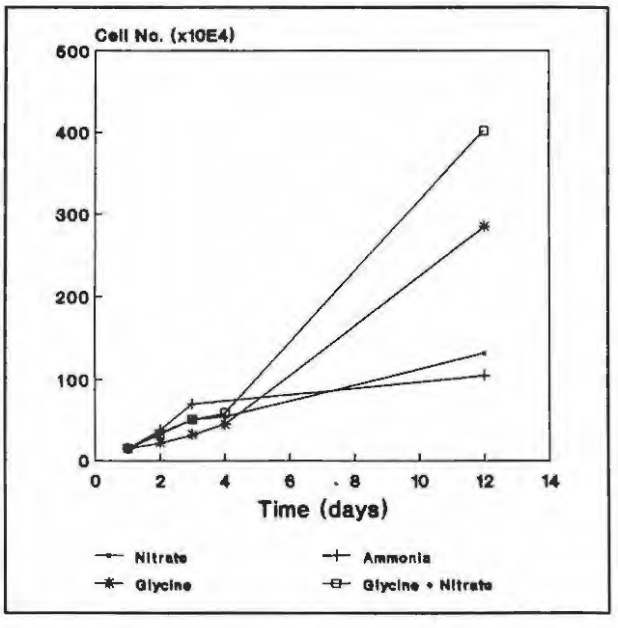


Figure 5.1 Growth of *D.salina* on different sources of nitrogen. Washed cultures were transferred to ASW medium containing as the sole source of nitrogen; 1mM KNO_3, 1mM NH_4Cl, 1mM glycine, and 0.05mM each of glycine and KNO_3.

Table 5.1 Ammonium present in the culture medium at the start and at day 12 of the growth study as reported in figure 5.1.

	Day 1 ($\mu g.mL^{-1}$)	Day 12 ($\mu g.mL^{-1}$)
ASW + 1mM KNO_3	0.0	160
ASW + 1mM Glycine	310	450
ASW + 0.5mM KNO_3/Gly	160	330

The simultaneous appearance of ammonium in the medium shown in table 5.1 indicated that an active process of deamination had taken place and that this was both rapidly activated and accentuated where glycine was the sole nitrogen source.

The amount of amino acid in the medium (in the mM range) had a direct bearing on cell productivity. The results in figure 5.2 showed an optimum of between 1 and 10mM where glycine was used as the sole nitrogen source. At concentrations above 10mM the glycine stimulation effect was reduced.

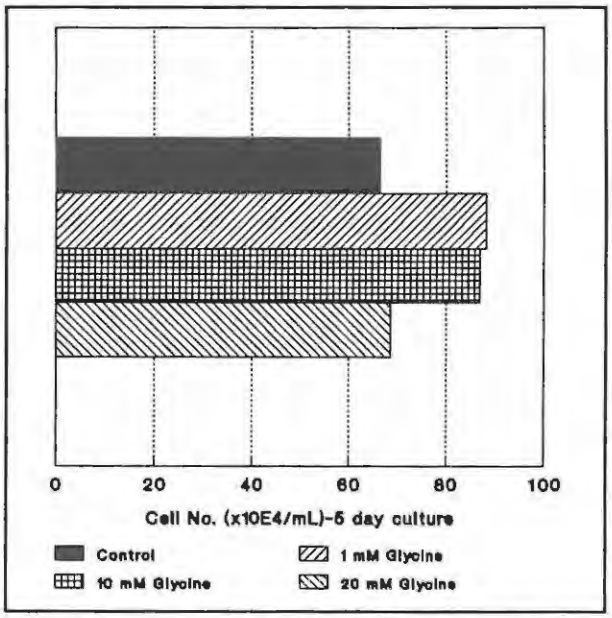


Figure 5.2 The effect of glycine concentration on the growth of *D.salina*, where glycine was used as the sole nitrogen source.

5.4.2. Deamination and Direct Uptake.

To determine whether the appearance of ammonium in *D.salina* cultures incubated with 5mM glycine could be attributed to the presence of inducible deaminases on the algal surface (recently reported for *Chlamydomonas reinhardii* by Munoz-Blanco *et al.*,1990) cells were incubated with cycloheximide and chloramphenicol. The results recorded in table 5.2 showed a continuous removal of glycine in the presence of cycloheximide, but its inhibition where the cultures were incubated with chloramphenicol. This result indicated that at high levels of

glycine addition (mM range), bacterial activity was largely responsible for its removal via the deamination, which resulted in the accumulation of ammonium observed in table 5.1. The presence of surface deaminases can be provisionally excluded for *D.salina*.

Table 5.2 The effect of cycloheximide ($4\mu g.mL^{-1}$) and chloramphenicol ($50\mu g.mL^{-1}$) on the removal of glycine (5mM) from the medium of a *D.salina* culture.

	Day 1	Day 2	Day 3	Day 5
Glycine (mM)	5.1	4.15	2.91	0.51
Gly + Cyclohex.	5.1	4.43	3.83	0.74
Gly + Chloramp.	5.3	5.0	4.89	4.76

The question of whether the glycine growth stimulation effect observed is due entirely to accumulated ammonium or could be attributed, in part at least, to the direct uptake of the amino acid, was resolved by the [^{14}C]-glycine labelling study reported in figure 5.3a. The results showed the rapid uptake of [^{14}C]-glycine by *D.salina* cells with its concomitant removal from the medium. The process was largely irreversible with only 17% of the label lost by exchange against 5mM glycine where this was measured 5 minutes after exposure to test conditions. Where cells were extracted after 24 hours exposure to the labelled glycine, 100% of the count was found to be incorporated into cellular protein. This result reflects the uptake of glycine in the picomolar range. When the experiment was repeated together with 5mM unlabelled glycine (figure 5.3b), the total label uptake was substantially reduced but the trend remained comparable. In this case 86% of the label taken up was incorporated into algal cell protein. Both the competitive inhibition, by unlabelled glycine, and the degree of irreversibility of label uptake indicated by these results suggested the presence of glycine binding sites on the surface of *D.salina*.

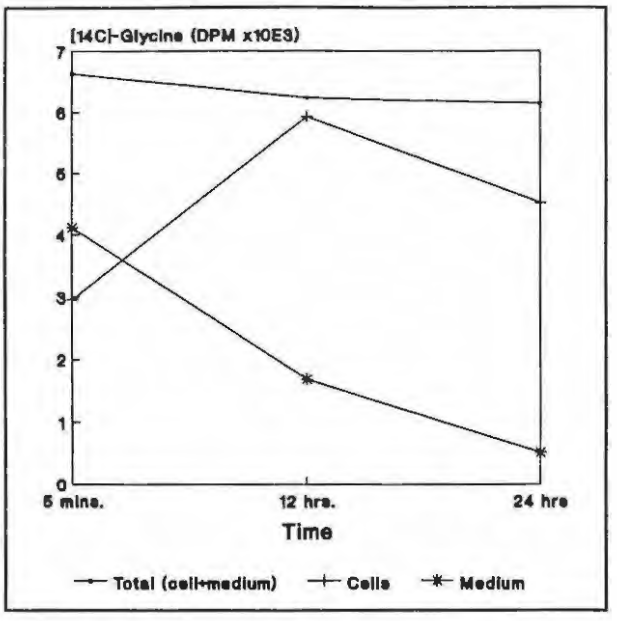


Figure 5.3a The uptake of [^{14}C]-glycine by *D.salina* as the sole nitrogen source in nitrogen-free ASW medium.

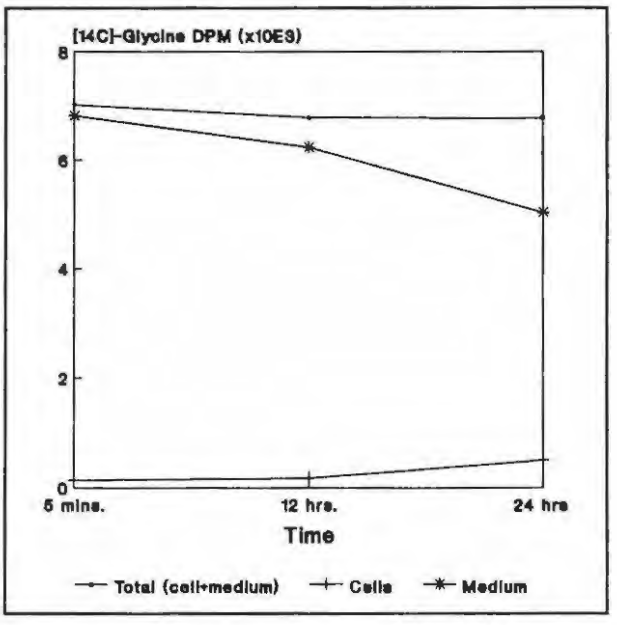


Figure 5.3b The uptake of [^{14}C]-glycine by *D.salina*, as described for figure 5.3a, with the addition of 5mM unlabelled glycine.

5.4.3. Chlorophyll Fluorescence.

The use of chlorophyll fluorescence as an indicator of primary photochemistry is well established (Ogren,1990) and will be discussed further in Chapter 8. The rapid quenching of fluorescence observed in *D.salina* cultures, within seconds of the addition of 1mM glycine, is shown in figure 5.4. The result indicated that the addition of glycine induced a sudden demand for reducing power supplied by photosystem II and hence suggested the presence of an active transport uptake process. Since the cells were suspended in standard ASW medium containing 5mM KNO_3, a level assumed to be saturating for nitrate uptake, it seems that the constitutive

presence of separate binding sites and/or uptake mechanism for glycine could be suggested for the cell surface of *D.salina*.

A similar response to that observed above was found with the addition of casamino acids, suggesting that uptake may not be limited to glycine alone. The results reflect a number of repetitions of these experiments (see figure 8.11). Antia *et al.* (1991) note that at least three different transport systems for amino acid uptake have been identified in the micro-algae.

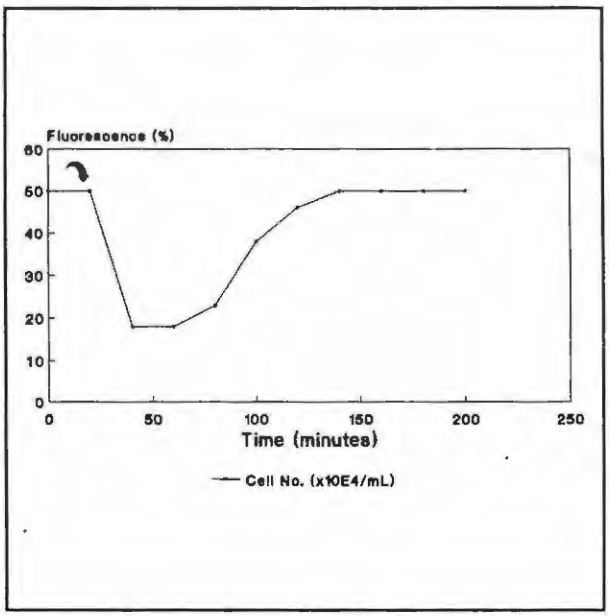


Figure 5.4 The quenching of chlorophyll fluorescence in a *D.salina* culture with the addition of 1mM glycine. Arrow indicates the time at which glycine was added.

5.4.4. Bovine Serum Albumin Uptake.

The uptake of bovine serum albumin (BSA) by *D.salina* was demonstrated with the addition of [^{14}C]-methylated BSA. The accumulation of the label in the cellular fraction is shown in figure 5.5.

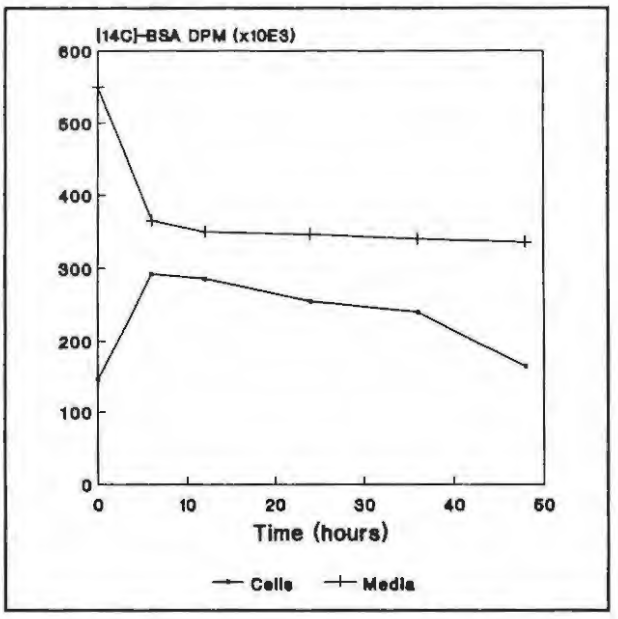


Figure 5.5 The uptake of [^{14}C]-methylated BSA by *D.salina*.

5.4.5. Glycerol Release and Heterotrophic Activity.

Glycerol was found to occur in the growth medium of all *D.salina* cultures, where it was measured. Figure 5.6a shows that the addition of glycine stimulated the release of glycerol by the organism, with the increase in chloramphenicol+glycine release being about twice that observed in the untreated controls, after 24 hours of exposure. The glycine enhancement of glycerol release, however, was not continuous and by 144 hours similar glycerol levels were measured in the medium of both control and glycine treatments. The presence of chloramphenicol was required to demonstrate this effect, indicating that the additional glycerol production was largely removed by the activity of the bacterial population. This observation was further supported by the increase in bacterial numbers seen in figure 5.6b, which coincides with the largest removal of glycerol at 144 hours. It is interesting to note, however, that a 77% increase in bacterial cell numbers correlates with an increase in glycerol consumption of only 15%, comparing glycine treatment with the control. This seems to indicate that glycerol is not alone responsible and suggests a role for glycine, in addition to glycerol, in the stimulation of bacterial population growth observed.

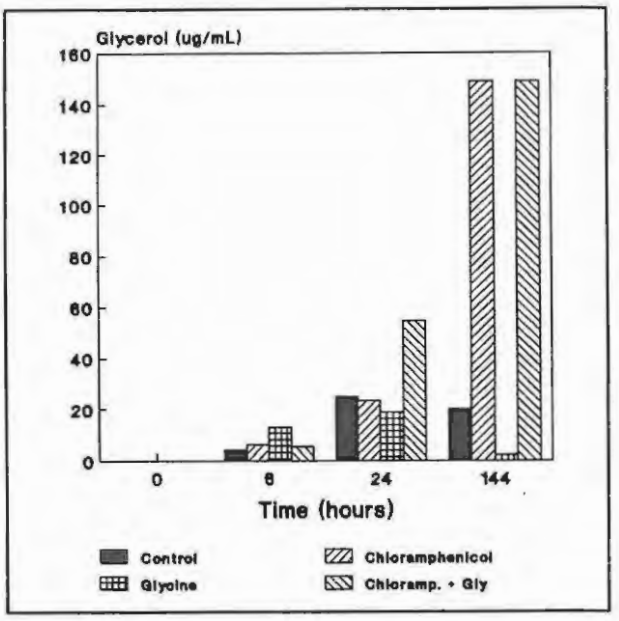


Figure 5.6a The release of glycerol by *D.salina*. A (5mM) glycine challenge is compared to controls with and without the addition of chloramphenicol (50μg.mL^{-1}).

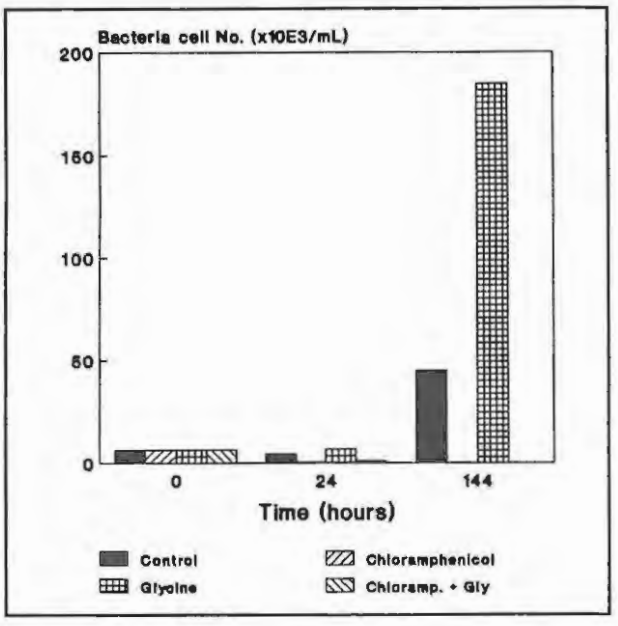


Figure 5.6b The change in bacterial cell numbers in response to glycerol release by *D.salina*.

Since the glycine addition appears to stimulate bacterial activity both directly, as indicated above, and indirectly, via an increased release of glycerol by *D.salina*, it would be important to clarify the nature of the association between the two populations where they occur in a nitrogenous medium such as the Hide Soak Liquor HROP. An apparent steady state condition is established with regard to ammonia accumulation and the question that arises is why bacterial deamination does not rapidly proceed to its end point, with toxic effects resulting in algal cell death. This occurs in the absence of algal growth as demonstrated in figures 4.2 to 4.5.

The enhancement of heterotrophic activity with the addition of glycerol was determined in an adapted algal/bacterial co-culture, derived from the batch operated HROP simulation, described in the previous chapter.

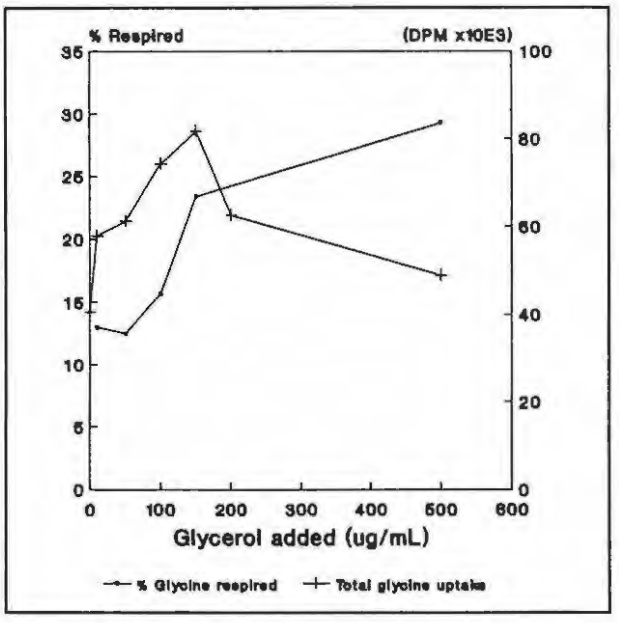


Figure 5.7 Total glycine uptake compared to the percentage respired.

Both glycine taken up and incorporated, and the total amount respired (measured as $^{14}CO_2$ released), was determined. The results in figure 5.7 show that with increasing levels of added glycerol, the amount of glycine respired increased continuously over the range evaluated. It can be assumed that respiration of the amino acid carbon skeleton also provides an indication of the equivalent availability of ammonia produced concurrently by deamination. The amount of glycine taken up by both the algal and bacterial components of the system rose, together with glycerol added, to a peak at 150μg.mL^{-1} and then declined again to approximately starting levels. Since the glycine uptake by the alga had already been shown to be nearly continuous (figure 5.3a,5.3b) and the quantitative role of bacteria in the deamination process had been demonstrated, it seems reasonable to assume that the uptake pattern observed here indicates the major role of the bacterial component in this effect. The trends observed in figure 5.7 seem to indicate that as glycerol levels rise, the bacteria rely less on the incorporation of the glycine carbon skeleton into cell structures and more on its direct respiratory breakdown as an energy source.

The relative heterotrophic potential of this system was measured with respect to glycine, by determining the kinetics of the process from a Lineweaver-Burke plot of the experimental data. In this case, the measurement includes both the respired and the incorporated uptake fractions. A Maximum Utilization Velocity, Vmax = 4.6μgC.L^{-1}.hr^{-1}, was measured before the addition of glycerol. With the addition of 50μg.mL^{-1} glycerol, utilization increased 44% to Vmax = 8.2μgC.L^{-1}.hr^{-1}. The turnover time for glycine was reduced from 5.6 to 2.5 hours. These results confirm the observation by Billen (1984), that the deamination rate in mixed

algal/bacterial systems is partly controlled by the C:N ratio. It is apparent that this is, in turn, partly under algal control.

5.5. DISCUSSION.

A demonstration of the uptake and utilization of organic compounds by *D.salina* not only presents a different perspective on what has until recently been considered an obligate photoautotroph, but also holds important implications for the integration of halophilic algal production and organic waste treatment in a saline system.

The uptake of both glycine and BSA has been demonstrated, with the amino acid internalised, possibly by an energy-requiring active transport process. The result accords with the observation by Ricketts (1988) that in a natural environment the micro-algal cell would need to be able to alter its nitrogen uptake from a widely fluctuating supply. The quantitation of direct glycine use, compared to the uptake of ammonium and other nitrogen sources was not determined, but a nutritional advantage was demonstrated for *D.salina* with the combination of glycine and inorganic nitrogen in the growth medium.

The enhancement of *D.salina* cell growth, where glycine is available, may partly account for the similar effect observed with the use of Hide Soak Liquor (see figure 3.1). The result certainly indicates a potential application for amino acid supplementation of defined inorganic media used in the biotechnological production of *D.salina*. The resulting enhancement of cell production could have a significant effect on the economics of the process.

The finding that ammonium accumulates in the medium largely as the result of bacterial deamination, confirms the source of the ammonium toxicity effect in tannery effluents identified in Chapter 3. The role of glycine in enhancing glycerol release by *D.salina*, and this in turn increasing the rate of glycine deamination by bacterial action, and hence ammonium availability, suggests the presence of a control mechanism operating between the algal and bacterial populations. The question why high rates of bacterial deamination are not maintained, resulting in the accumulation of ammonia to levels lethal to the alga, may be partly answered by the observation, already noted, that the size of the bacterial population is under algal control by an unknown mechanism of inhibition. It is now apparent from this study that a form of positive control also operates, with the induction of bacterial metabolic activity by an algal regulation of photosynthate release. While the results recorded here appear to show that the rate of glycerol release is under algal control (figure 5.6a), further studies demonstrating an ammonium concentration-controlled termination of glycerol release would be required to confirm the presence of a feed-back component to the mechanism.

An implication of this observation applied to the operation of the HROP, is that the system should be optimised for the algal control of the deamination rate and thus also of ammonia toxicity where proteinaceous wastes are to be treated.

The glycerol production potential of the system is substantial, and a rough calculation from the results acquired (accepting possible scale-up invalidation and the tentative nature of this type of extrapolation), indicates that an annual leakage of glycerol under similar conditions, would be in the range of about 6000tons.km^{-2}. The organic carbon could be made available to enhance degradation rates of organics fed to the saline HROP. This raises the interesting prospect of applying the saline HROP concept to the wider purpose of a waste disposal process for problematic refractory organic solids such as the secondary sewage sludge solids. Ponded disposal brines derived from the desalination of brackish water and the segregation of saline effluents (noted in Chapter 1) could be used to sustain a large-scale saline HROP, providing a utility for the waste during the course of its own disposal. This concept will be elaborated further in Chapter 10.

CHAPTER 6

THE ULTRASTRUCTURE OF EFFLUENT-GROWN *D.SALINA*

Summary.

Mechanisms that could account for the uptake and discharge of large molecular weight organic molecules were observed in ultrastructural study comparing Hide Soak Liquor-grown *D.salina* with ASW-grown controls. Growth in the effluent was found to be associated with the accumulation of multi-component vacuoles in the cytoplasm and a proliferation of the dictyosome/endoplasmic reticulum apparatus, further suggesting the presence of an uptake and release process. A role for the glycoprotein, glycocalyx in membrane-linked events associated with the hypersalinity stress response was noted, and a previously unreported function for chloroplastic vacuoles has been suggested.

6.1. INTRODUCTION.

The study that follows was undertaken to see whether ultrastructural changes could be observed in effluent-grown *D.salina* which could further clarify the observations suggesting the uptake and utilization of organic components present in Hide Soak Liquor. Both the uptake and release phenomena are controversial issues on which the available evidence is conflicting and is important to an understanding, and possibly the operation, of a *D.salina* based HROP process for treating saline organic effluents.

The *Dunaliella* genus has been the subject of a number of general ultrastructural studies, pertaining to *D.salina* (Trezzi *et al.*,1964;Vladimirova,1978); *D.bioculata* (Marano&Izard,1968;Marano,1976); *D.tertiolecta* (Hoshaw&Maluf,1980); *D.primolecta* (Eyden,1975;Hyams&Chasey,1975); *Dunaliella sp.* (Berkaloff,1966;Peterfi&Manton,1968;Werz&Kellner,1970).

Olivera and Huynh (1989) have reported ultrastructural changes in *D.tertiolecta* grown in the presence of hypoxanthine compared to nitrate as a source of nitrogen. Antia *et al.* (1991) report a study by Klut who investigated an ultrastructural mechanism for the internalization of organic macromolecules, also by *D.tertiolecta*. The evidence points to the presence of a recepto-mediated pinocytosis with protein accumulating in micro-bodies and phosphatase-positive vacuoles associated with the endoplasmic reticulum.

A number of ultrastructural studies have indicated a mechanism of release that could account for the contending observations concerning the appearance of organics in the medium of *Dunaliella* cultures. Hoshaw and Maluf (1981) demonstrated the presence in *D.tertiolecta*, of tubular or vesicular-like evaginations of the cytoplasmic

membrane which appeared to be pinched off into the surrounding medium. These proliferated with increasing osmotic stress and the authors suggested that they may allow the release of organics without changes in membrane permeability. Maeda and Thompson (1986) and Einspahr *et al.* (1988a) have demonstrated a role for these vesicles in providing additional membrane material to allow for the rapid cell expansion observed on hypo-osmotic shock. These authors have also suggested vesicular fusion with the membrane as a possible way to account for glycerol release.

Dunaliella is known to excrete organic material other than glycerol to the surrounding medium (Oliviera *et al.*, 1980) and these authors have demonstrated the presence of glycocalyx cell coat composed of a glycoprotein in *D.tertiolecta*. They suggest a dispersal of cell coat material may occur, especially under conditions of stress, and that an equilibrium condition exists between the tendency of the surface coat to be desorbed into the medium and adsorbed to the cell surface. These authors have suggested that this production of glycocalyx may contribute to the accumulation of organic compounds in the medium.

6.2. RESEARCH OBJECTIVES.

An attempt was made to link ultrastructural changes in *D.salina* to specific physiological response phenomena and enable a speculative conclusion concerning uptake and release mechanisms. The following questions were identified:

1. Does the previously reported ultrastructural evidence that *D.tertiolecta* is able to utilize organic nitrogen apply equally to *D.salina*, and are the organics present in Hide Soak Liquor utilized;
2. Can the vesicle theory of glycerol release by *D.salina* be substantiated by correlating the presence of these substrates with a number of physiological states, including hypo- and hypersalinity stress, stationary growth phase and elevated temperature;
3. Can the presence of a glycocalyx be observed in *D.salina* and can its occurrence be correlated with changes in the above conditions?

6.3. MATERIALS AND METHODS.

6.3.1. Culture

Cultures of *D.salina* var *bardawil* (CCAP 19/30) were grown under conditions previously described. Cultures were centrifuged at 3000g for 10 minutes at 10°C and resuspended in an equivalent volume of fresh ASW medium. Salinity stress was applied by adjusting the medium to 3.5M NaCl.

6.3.2. Entrapment and Embedding

Samples were prepared for electron microscopy following the methods of Cross (1979). Cells were centrifuged at 3000g for 10 minutes at 10°C and the pellet immediately entrapped in 6% agar (Difco) held at 48°C and then

rapidly cooled in an ice bath. Soon after solidification, the agar pellet was cut into 2mm cubes and fixed overnight in 2.5% glutaraldehyde in 0.1M phosphate buffer.

Samples were then washed in 0.1M phosphate buffer followed by post-fixation for 90 minutes in 1% phosphate buffered osmium tetroxide. Following further buffer washes the samples were dehydrated by transfer through a series of ascending concentrations of ethanol (30%-100%). This was followed by two washes of propylene oxide and transition to a resin medium through three propylene oxide:epoxy resin mixtures (75:25,50:50, 25:75) and finally pure epoxy resin. Samples were then transferred to fresh epoxy resin in embedding moulds, and polymerization was allowed to take place over 30 hours at 60°C.

6.3.3. Sections

Ultra-thin sections were cut using a LKB 111 ultramicrotome and collected onto alcohol-washed grids after which they were post-stained with 5% aqueous uranyl acetate (30 minutes), followed by Reynold's lead citrate.

6.3.4. Transmission Electron Microscopy (TEM)

Grids were examined with a JEOL JEM 100 CK11 TEM at an accelerating voltage of 80kV. Processes for preparing algae for EM have been described by Reimann *et al.* (1980).

6.3.5. Morphometric Analysis

Morphometric analysis (stereology) was undertaken following the methods of Weibel (1969), and as described in Fagerberg (1980). A regular lattice grid (distance between points d=5mmx4600) was superimposed on electron micrographs. Volumes were obtained from the formula:

$$Sv = 2It/k.d.Pt$$

where k = 2(lattice grid)
d = correction to actual magnification
Pt = the total number of grid points within the cell area
It = the total intersections within the lattice grid.

6.3.6. Scanning Electron Microscopy (SEM)

Cells were entrapped in agar as previously described and cubes plunge-frozen in sub-cooled nitrogen (-200°C) in a Hexland CT 1000 cryo SEM device. The samples were tben transferred into the cryo preparation chamber of a Jeol JSM 840 SEM and fractured. Specimens where then either:

1. observed un-coated on the cryo-stage at -150°C or,
2. allowed to etch by sublimation of ice at -80°C, sputter-coated with gold and observed on the cryo-stage at -150°C.

6.3.7. Glycerol Assay.

Glycerol was assayed using enzyme analysis (Boehringer).

6.4. RESULTS

The ultrastructural study of *D.salina* grown in Hide Soak Liquor showed a number of morphological changes compared to ASW medium-grown controls (figure 6.1).

6.4.1. Cell Surface

The cell surface in effluent-grown cells acquired an undulating appearance (figure 6.1B) with pit-like invaginations (p) of the cytoplasmic membrane (Cm) (figure 6.2A,B,D,E). Neither feature was observed in any of the other treatments evaluated. Certain of the pits were either coated or partially filled with electron dense material.

A space containing large numbers of vesicles (Ve) and mitochondria (M) was observed to develop between the cytoplasmic membrane and chloroplast (Ch) in effluent-grown cells (figure 6.2A). This area, which is not differentiated to the same degree in ASW-grown controls, appears to receive the contents of the pinocytotic invaginations. Multi-vesicular micro-bodies (Mb) occur in the region of the endoplasmic reticulum (Er) (figure 6.2C,6.3C).

6.4.2. Endoplasmic Reticulum

A close association exists between the endoplasmic reticulum, the nucleus (N) and the dictyosome (D) located in the anterior region of all cells examined (figure 6.3A,B,C). The *trans* face of the dictyosome is located close to the anterior tip of the chloroplast and to the cytoplasmic membrane.

A substantial proliferation of the endoplasmic reticulum was found in effluent-grown cells (figure 6.3B,C) which corresponds with the observation by Olivera and Huynh (1989) in *D.tertiolecta* grown with hypoxanthine. This structure showed an unusual proliferation to the *trans* or forming face of the dictyosome. The entire region was characterized by large vacuoles (Va) and copious amounts of electron dense, membranous-type material.

The area between the dictyosome region and the chloroplast sinus, in effluent-grown cells, was filled with multi-component vacuoles (figure 6.4A,D). The number of cytoplasmic vacuoles increased nearly two-fold in effluent-grown cells (figures 6.1B,6.8) and these structures are filled with membranous, granular material. Electron dense masses also occur, possibly polyphosphates (Pp) (Bochem&Sprey 1979), and in addition crystalline material, which on sectioning resulted in the repeated tearing of the vacuolar regions.

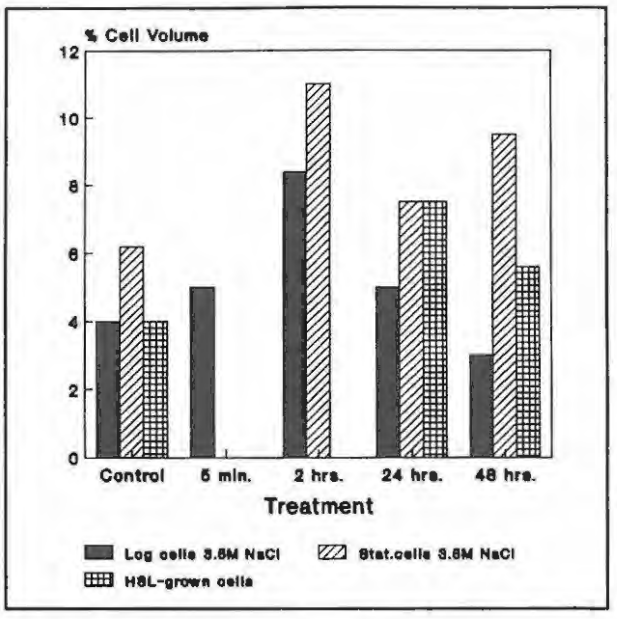


Figure 6.8 Morphometric measurement of changes in cytoplasmic vacuoles as a percentage of cell volume. Results compare salt stressed log and stationary phase cells and Hide Soak-grown cells of *D.salina*.

The ultrastructural pattern observed in effluent-grown cells is distinctive and suggests the presence of an uptake and utilization process. Antia *et al.* (1991) has reported similar ultrastructural modifications in *D.tertiolecta* grown in the presence of protein. Note the change in the vacuole percentage of cell volume that is evident during hypersaline shock.

6.4.3. Exocytosis

In addition to the endocytotic pits, large invaginations of the membrane were also observed, which have not been reported in previous studies, and indicate the presence of either an engulfment or the release of particulate matter (E1,2,3) (figures 6.1B,6.2A,C,6.4C,D).

The invaginations are associated with membranous and vacuolar material similar in appearance to the contents of the cytoplasmic vacuoles described above. Diffuse accumulations of membranous material is noted in the immediate vicinity of these cells (figure 6.4D). This, together with the following sequence of observations, tends to suggest the operation of an exocytotic, extrusion process, whereby accumulated vacuolar contents are released to the cell's external environment.

Three possible stages can be noted and have been termed exocytosis 1 (E1) in figures 6.2A and 6.4A, where vacuoles leave the chloroplast sinus area and press against the cytoplasmic membrane; exocytosis 2 (E2) in figure 6.1B, and exocytosis 3 (E3) in figures 6.2A,C, and 6.4C,D.

6.4.4. Chloroplast

Thylakoid (T) stacking was less pronounced in effluent-grown cells showing a diffuse and disturbed arrangement within the chloroplast (figures 6.1A,B,6.2B).

Mitochondria appeared within the chloroplasts of these cells which was rare in ASW-grown controls (figure 6.2A,B). These mitochondria showed an increased luminal area (also noted by Olivera&Huynh,1989), and they were enclosed in a double membrane, sometimes more than one per sacculus. This could indicate enhanced respiratory activity within the chloroplast.

The pyrenoid area (Py) decreased size in effluent-treated cells, and exhibited diffuse and less well-defined margins and a disturbance of the neat circular arrangement of starch granules found in ASW-grown controls (figure 6.2B,F).

These observations, together with the recent location of Rubisco (ribulose 1.5-bisphosphate carboxylase/oxygenase) in the pyrenoid area of the algae (McKay&Gibbs,1991), suggest a reduction in the photosynthetic activity of the chloroplast region of effluent-grown cells.

The clear zone between the pyrenoid/starch granules (S) and the rest of the chloroplast, here termed the peri-pyrenoidal space (Pps) (figure 6.1A), decreased by >50% cell volume in effluent-grown cells. The significance of this change is uncertain. It was found that this area also showed a reduction in size in response to hypersaline shock, with the recovery of the original volume substantially completed within 24 hours of the stress (figure 6.9). The change, however, was not immediate and developed over a period of two hours, suggesting that this area may be associated with the mobilization of the glycerol osmoticant. This tentative conclusion is further supported by the observation in figure 6.8, showing the reverse of this trend for vacuole volume. The possibility that the peri-pyrenoidal contents may be mobilized and transferred to the vacuoles during hypersaline stress is both intriguing and requires further evaluation. It is significant, however, that stationary phase cells do not show a reversal of the phenomenon, and changes were permanent through to the 48 hour observation. It is known that *Dunaliella* grown under conditions of high nutritional nitrogen show a shift in metabolism away from the storage of carbohydrates (Semenenko&Abdullaev,1980).

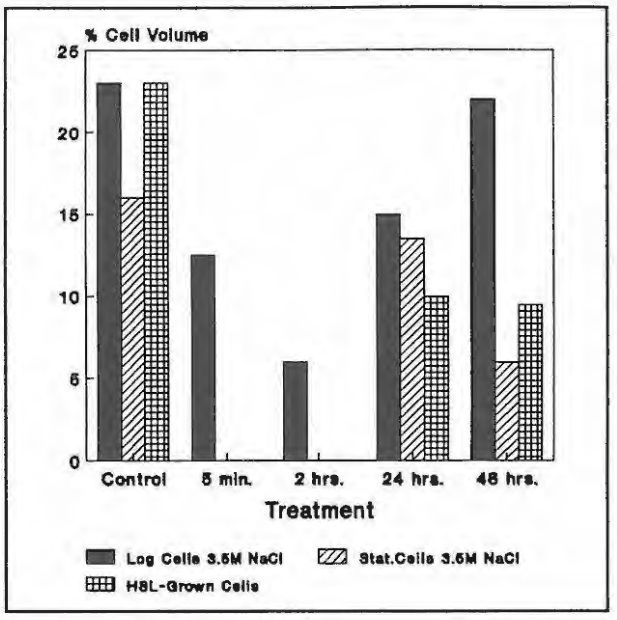


Figure 6.9 Morphometric measurement of changes in the size of the peri-pyrenoidal space comparing hypersaline stressed log and stationary phase *D.salina* and Hide Soak-grown cells. Measurement reflects a percentage of cell volume.

6.4.5. Freeze Fracture.

The observation of uncoated, freeze fractured specimens of ASW-grown *D.salina* was undertaken in an attempt to view the cell in as near-natural a condition as is feasible, and thus possibly visualise the peri-pyrenoid area. The results showed the surprising presence of distended vacuoles or compartments distributed throughout the chloroplast region (figure 6.5). These apparently turgid chloroplastic vacuoles (Cv) with condensed material in the centre have not been previously reported. Ice crystal damage was not observed. It can be speculated that this observation represents the natural state and that the vacuoles are comparable to the interthylakoid spaces observed on fixed and stained TEM sections (figure 6.1A).

The function of these structures is obscure, but it is possible that they may be similar to, or an extension of, the peri-pyrenoidal space.

6.4.6. Mechanisms for the Release of Organic Components.

A possible mechanism for the release of certain organic constituents including glycerol and protein by *D.salina* has already been noted.

In ASW-grown cells smaller vacuoles were noted close to the membrane surface and were observed to be discharging their contents by extrusion rather than by an invaginating exocytosis. These cells produce release vesicles (Rv) (figures 6.4B,E,F,6.6A,B). A pinching-off type process may be involved (Hoshaw&Maluf, 1981) and small vacuoles do occur in the immediate vicinity of these vesicles (figure 6.6C). Structures in figure

6.6C,D, suggest that the vacuolar extrusions may also burst, releasing their contents free of any membranous coating layer, directly to the cell exterior.

In addition to the above, the presence of glycocalyx-associated (G) release vesicles (RV) were found which were not linked with underlying vacuoles or distortions of the cytoplasmic membrane. These were present as an apparent ballooning of the glycocalyx layer which was particularly evident in 3.5M NaCl-stressed cells (figure 6.6E). This appears to indicate the entrapment, in the glycocalyx, of material discharged by diffusion across the cytoplasmic membrane.

An examination of freeze-fractured, gold coated specimens, was used in an attempt to visualise extrusion vesicles in fresh cells that had not been subjected to the possible distortions of the fixing, staining and sectioning processes. The results, while not entirely convincing, do show the presence of numerous small pores in the outer cytoplasmic membrane that could be associated with the presence of vesicles (figure 6.6F).

6.4.7. Glycerol Release

An attempt was made to visualise ultrastructural changes that might accompany an enhancement of glycerol release by the manipulation of temperature, as demonstrated by Wegmann *et al.* (1980).

Cells grown at 38°C for 24 hours showed a 300% increase in glycerol release, compared to salt-stressed controls (figure 6.10). No commensurate increase in extrusion vesicle numbers was noted, as previously reported by Hoshaw and Maluf (1981). The ultrastructural results, after growth at 38°C for 5 days were both dramatic and bizarre.

The cytoplasm showed comprehensive granulation consisting of a proliferation of what appear to be ribosomes, suggesting large-scale protein synthesis (figure 6.7A-D). A striking observation was the massive over-production of the glycocalyx coat, which accumulated as a thick layer around the cell (figure 6.7A,B,D). The layer had been sloughed off and in places numerous successions of disposed layers could be observed. The glycocalyx appeared to possess a well-defined integrity, with consolidated membrane-like characteristics that persisted after sloughing. It did not appear as a diffuse jelly-like layer dispersing to the medium, as previously reported by Olivera *et al.* (1980).

The reaction to the stress conditions appears as a substantial re-direction of cellular activity towards the synthesis of the glycoprotein coat. The correlation with glycerol release, and the absence of increased numbers of extrusion vesicles in these cells, indicates that glycerol is lost by diffusion across the cytoplasmic membrane. The overproduction of the glycocalyx suggests a role for cytoplasmic membrane-associated glycoprotein in the regulation of membrane permeability and also in the hypersaline stress response, as has been recently suggested by Sadka *et al.* (1991).

The chloroplast showed a large accumulation of β-carotene (bc) droplets and the pronounced stacking of thylakoid membranes (T) (figure 6.7C).

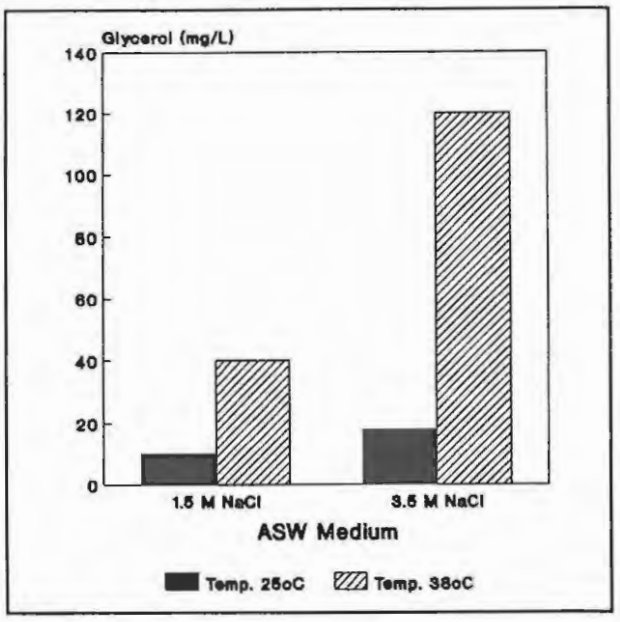


Figure 6.10 Glycerol release by *D.salina* under unstressed (1.5M NaCl) and hypersalinity-stressed (3.5M NaCl) conditions comparing the response at 25°C and 38°C.

6.5. DISCUSSION.

D.salina cells, grown in Hide Soak Liquor, show a number of noteworthy ultrastructural changes compared to ASW-grown controls and the data acquired in this study allows a speculative interpretation of the processes involved. The changes observed were similar to those recently reported in *D.tertiolecta* grown in the presence of hypoxanthine (Olivera&Huynh,1989). Endocytotic pockets or pits were apparent in the cytoplasmic membrane, often in association with tiny vesicles massed immediately below it. The role of similar vesicles in the extension of membrane area, that would be required for the observed endocytotic activity, has been the subject of reports by Maeda and Thompson (1986) and Einspahr *et al.* (1988a). The labelled BSA study reported in Chapter 5 indicates that both binding and uptake of bovine protein present in Hide Soak Liquor does occur. Additional mitochondria noted in the endocytotic area suggest the function of an energy-requiring process not present in control cells. These data, together with the presence of multi-vesicular bodies, strongly suggests the presence of a receptor-mediated pinocytotic (endocytotic) uptake mechanism in *D.salina* for the internalization of large molecular weight organic molecules. Further immunocytochemical studies are currently being undertaken to confirm this point.

Whether the incorporated molecules serve a nutritional function in the organism is not yet clear and the previous investigators were surprisingly unable to show the enhancement of growth that has been demonstrated for Hide Soak Liquor in this study. While an unequivocal demonstration of a role for organics in producing this effect

has not been provided here, a number of indications have emerged from this study which strongly suggest that this might be the case.

The unusual proliferation of the endoplasmic reticulum on both the *cis* and *trans* faces of the dictyosome was also observed by Olivera and Huynh (1989), and the substantial accumulation of vacuoles containing a range of components in the cytoplasm is indicative of intense activity compared to ASW-grown control cells. Dictyosome vesicles are known to be responsible for delivering cell wall material to specific regions of the plasma membrane in plant cells, and this has been demonstrated in *D.salina* by Maeda and Thompson (1986). Einspahr *et al.* (1988a) have shown in *D.salina* that the endoplasmic reticulum serves as a reservoir of membrane material for accommodating cell expansion during hyposalinity shock. Lysosomal activity has been attributed to these vacuoles (Antia *et al.*,1991), and Marty *et al.* (1980) have pointed out that this apparatus in plants is known to play a major role in the recycling of building blocks that result from the digestion of macromolecules. Tischner (1984) has demonstrated an interaction between the chloroplast, cytoplasmic vacuoles, and the regulation of nitrogen metabolism in *Chlorella*, and Hoshaw and Maluf (1981) have suggested that the accumulation of these vacuoles, especially during stationary phase, may represent the break-down site of accumulated by-products.

The proliferation of membrane-enclosed mitochondria, of increased volume density in the chloroplast (possibly containing peroxidase activity), reduced thylakoid stacking and a smaller, more diffuse pyrenoid, suggests enhanced respiratory activity and a reduced reliance on photosynthetic productivity. Pfeifhofer and Belton (1975), Vladimirova (1978) and Hoshaw and Maluf (1981) have all showed increased thylakoid stacking in response to stress, which may also serve to indicate the absence of stress conditions in effluent-grown cells. The function of the pyrenoid as the site of Rubisco activity (ribulose 1,5-bisphosphate carboxylase/oxygenase) has been recently reviewed by McKay and Gibbs (1991). It also seems unlikely that a process involving such a major departure from the morphological norm of cells grown in inorganic medium, the full effect of which appears within 24 hours, should not afford the organism certain significant advantages. It would seem appropriate to conclude that, in its natural environment, it would be the exception rather than the rule for the organism to find itself in a completely inorganic medium, totally free of amino acids and large molecular weight proteins. However, the evidence for a nutritional role in the uptake process is not conclusive and further biochemical studies involving the incorporation of nitrogen and carbon skeletons of the ingested protein into cell components will need to be demonstrated.

This study has also shown the operation of a number of release mechanisms whereby *D.salina* may be able to transfer intracellular components to its external environment. The role of micro-vesicle fusion with the cytoplasmic membrane has been reported by Maeda and Thompson (1986), and Hoshaw and Maluf (1981) noted the presences of vesicles pinched off from the cell surface in *D.tertiolecta*. Aaronson *et al.* (1971) and Pickett-Heaps (1975) had suggested that these structures may account for the excretion of proteins and carbohydrates

by algal cells. The occurrence of similar structures in *D.salina* has been reported here. In addition, what appears to be a vacuolar exocytotic excretion process was observed in effluent-grown cells, a process which has not been previously reported in *Dunaliella*. Among the diverse components contained in the released vacuoles, large quantities of membranous material are present suggesting the fusion of a number of vacuoles prior to their release. The amalgamation and removal of defunct vacuoles would also seem to imply a nutritional role for these structures.

The various extrusion structures noted here have been suggested to account for the substantial release of glycerol that has been observed in the medium in which *Dunaliella* is grown (Hoshaw& Maluf,1981;Maeda&Thompson,1986). However, when the glycerol release process was accelerated by temperature elevation (Wegmann *et al.*,1980), no increase in the numbers of extrusion vesicles was observed, implicating a diffusional loss. However, a massive overproduction of the glycoprotein cell coat (glycocalyx) did occur, and conceding an attempt by the cell to overcome the substantially enhanced loss of photosynthate, a role for the overproduction of this structure in permeability function could be suggested. Catt *et al.* (1988) have shown that hydroxyproline-rich glycoproteins form the main structural components of the cell wall of *Chlamydomonas* and is especially noticeable in *D.salina* in stationary phase cells (Melkonian&Preisig,1984). Trick *et al.* (1983) reported the release of hydroxylamine-type substances by *D.tertiolecta*, and Antia *et al.* (1991) suggested that the sloughing of the glycocalyx may account for the protein and amino sugars that are reportedly released by *Dunaliella*. This result provides a convincing demonstration that such an effect does occur. Sadka *et al.* (1991) have recently reported the *de novo* synthesis of a 155kD glycoprotein in salt-stressed cells which, they have suggested, may be involved in osmoregulatory function and in the operation of extrusion pumps in the cytoplasmic membrane.

These observations provide further ultrastructural support for a link between the cytoplasmic membrane, the response to stress and its control in *D.salina*.

A further observation that emerged from this ultrastructural examination of *D.salina* was the presence, in uncoated freeze fractured samples of *D.salina*, of discrete, apparently distended vacuoles in the chloroplast that were not evident in TEM sections. Since a follow-up study of this phenomenon was not undertaken, any function attributed to these structures must necessarily be speculative. It seems, however, that the only area that could account for their presence is the interthylakoid region of the chloroplast. If these vacuoles contained fluids which are part of the osmoregulatory apparatus of the cell, their collapse in TEM sections would explain their absence in previous reports. Figure 6.9 shows that rapid changes occur in the area surrounding the pyrenoid in response to hypersalinity stress. The freeze fracture technique used here has apparently not been previously reported in ultrastructural studies on *Dunaliella*, and further evaluation is required to clarify this observation.

In conclusion, it seems that the data reported in this study are sufficient to establish a strong link between the growth of *D.salina* in Hide Soak Liquor, and its utilization of certain organic components present in this medium. An indication is provided that the organic removal function of a *D.salina*-based HROP would extend beyond a mutualistic association with the heterotrophic bacterial population. The process can be anticipated to function in a similar manner to that reported for the fresh water HROP, (Abeliovich,1986) where the algae are responsible for removing part of the organics present in the treated effluent. Cellular mechanisms which could account for this process appear to be present in *D.salina*.

Explanation of figures 6.1 - 6.7

Page 93: Figure 6.1 Near longitudinal sections of *D.salina* cells grown in ASW medium [A] and Hide Soak Liquor [B] and both in logarithmic growth phase. Notable differences in the effluent-grown cells include the irregular cytoplasmic membrane (Cm), exocytosis stage (E2) and the accumulation of vacuoles (Va) possibly containing polyphosphate (Pp) with anterior region between the nucleus (N) and the chloroplast (Ch). Other structures include the dictyosome (D), endoplasmic reticulum (Er), nucleolus (No), mitochondria (M), pyrenoid (Py), starch granules (s), β-carotene droplets and the sterigma "eyespot"(S) and peri-pyrenoid space (Pps). The speculative presence of chloroplast compartments or vacuoles (Cv) were noted.

Page 94: Figure 6.2 Sections through *D.salina* cells grown in Hide Soak Liquor ([A] to [F]). Membrane pits (p) and exocytotic stages (E1) and (E3) may account for uptake and release mechanisms. Note the accumulation of vesicles (Ve) and mitochondria (M) in the cytoplasmic space between the chloroplast and the cytoplasmic membrane (Cm). Membrane bound mitochondria (M.ch) occur in the chloroplast. The pyrenoid (Py) is more diffuse in effluent-grown cells. Vacuoles (Va) and multi-vesicular micro-bodies (Mb) occur in the anterior cytoplasmic region.

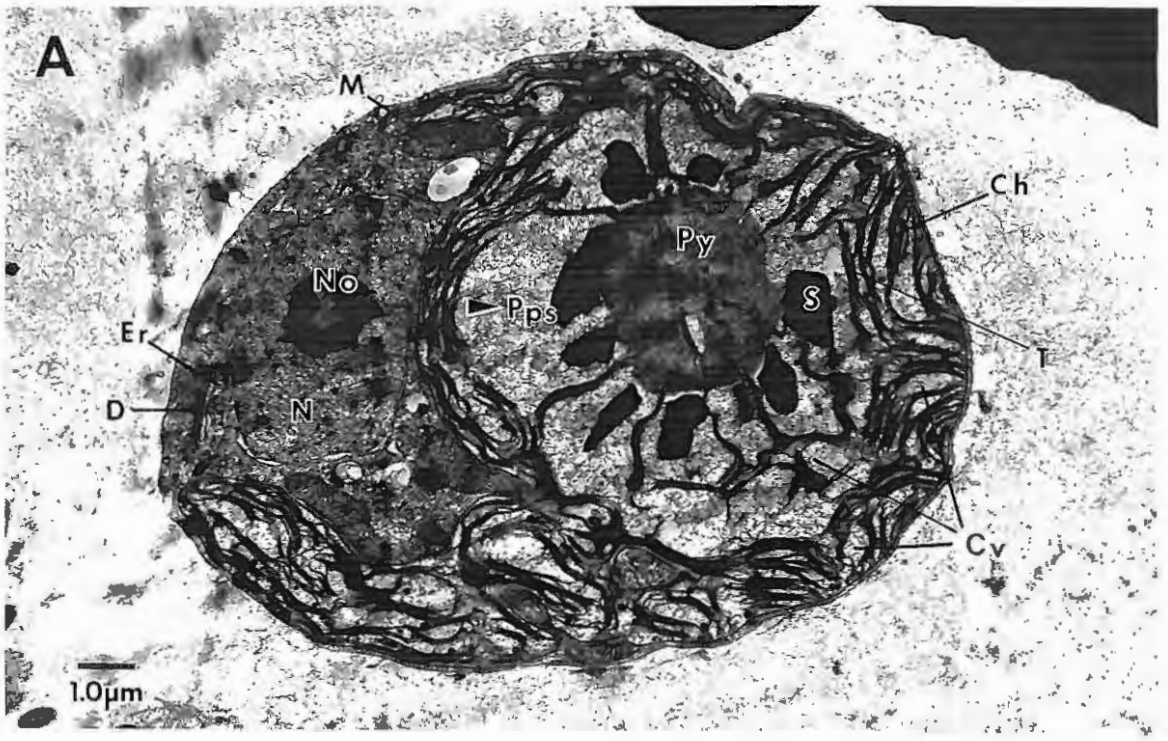
Page 95: Figure 6.3 Sections through the anterior cytoplasmic region of *D.salina* cells grown in Hide Soak Liquor ([A] to [E]). Note the large, well developed dictyosomes, the proliferation of the endoplasmic reticulum (Er) on both its *cis* and *trans* faces, vacuoles (Va), vesicles (Ve) and an accumulation of membranous material. Multi-vesicular micro-bodies (Mb) are also present.

Page 96: Figure 6.4 Sections through the anterior region of *D.salina* cells grown in Hide Soak Effluent ([A], [C] and [D]), showing the accumulation of multicomponent vacuoles (Va) and exocytosis stages (E1, E2 and E3). Note the accumulation of membranous material (Me) outside the cell. [B], [E] and [F] - ASW-grown cells showing release vesicles.

Page 97: Figure 6.5 Freeze fracture SEM of ASW-grown *D.salina* showing the presence of compartments or vacuoles (Cv) in the chloroplast (Ch). The structures are not present in the cytoplasmic region (C).

Page 98: Figure 6.6 Sections through ASW-grown *D.salina* cells, hypersalinity stressed to 3.5M NaCl ([A] and [B]). Vacuoles (Va) adjacent to the cytoplasmic membrane show the pinching off of release vesicles (RV). [C] and [D] - vacuoles appear to burst releasing their contents (Vr). [E] - vesicles accumulate in the glycocalyx layer. [F] - freeze etched, coated SEM of ASW-grown cells showing the possible presence of pores in the cytoplasmic membrane.

Page 99: Figure 6.7 Sections through *D.salina* cells grown in ASW medium at 38°C and subjected to hypersalinity shock at 3.5M NaCl ([A] to [D]). Note cytoplasmic granulation, the proliferation of the glycocalyx (G), the accumulation of β-carotene droplets (bc) and thylakoid stacking in the chloroplast.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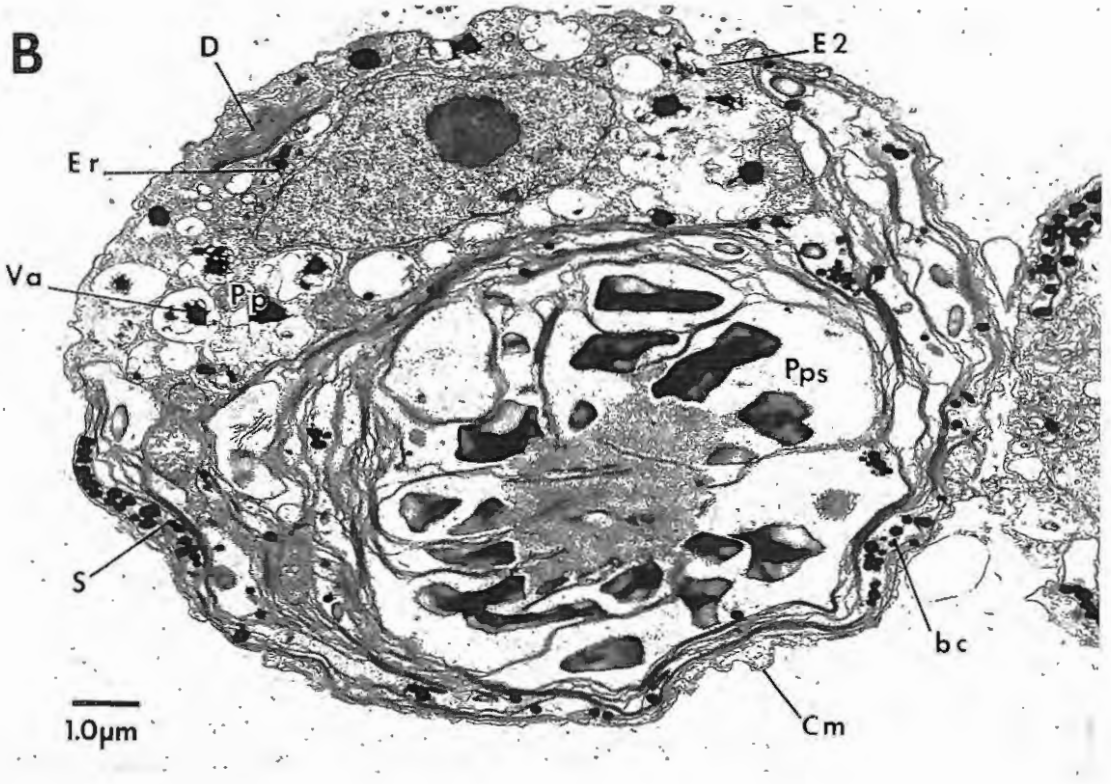
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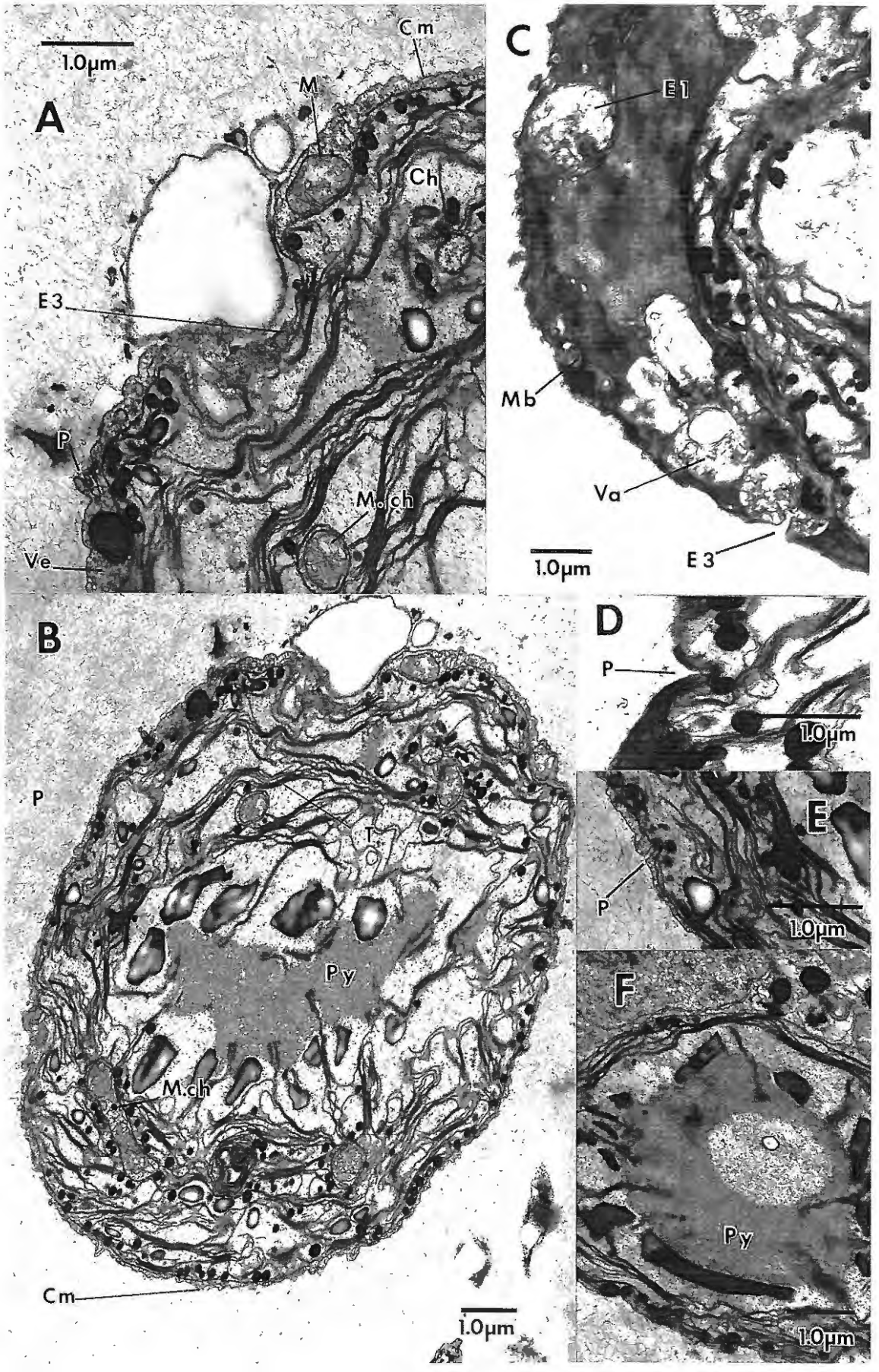
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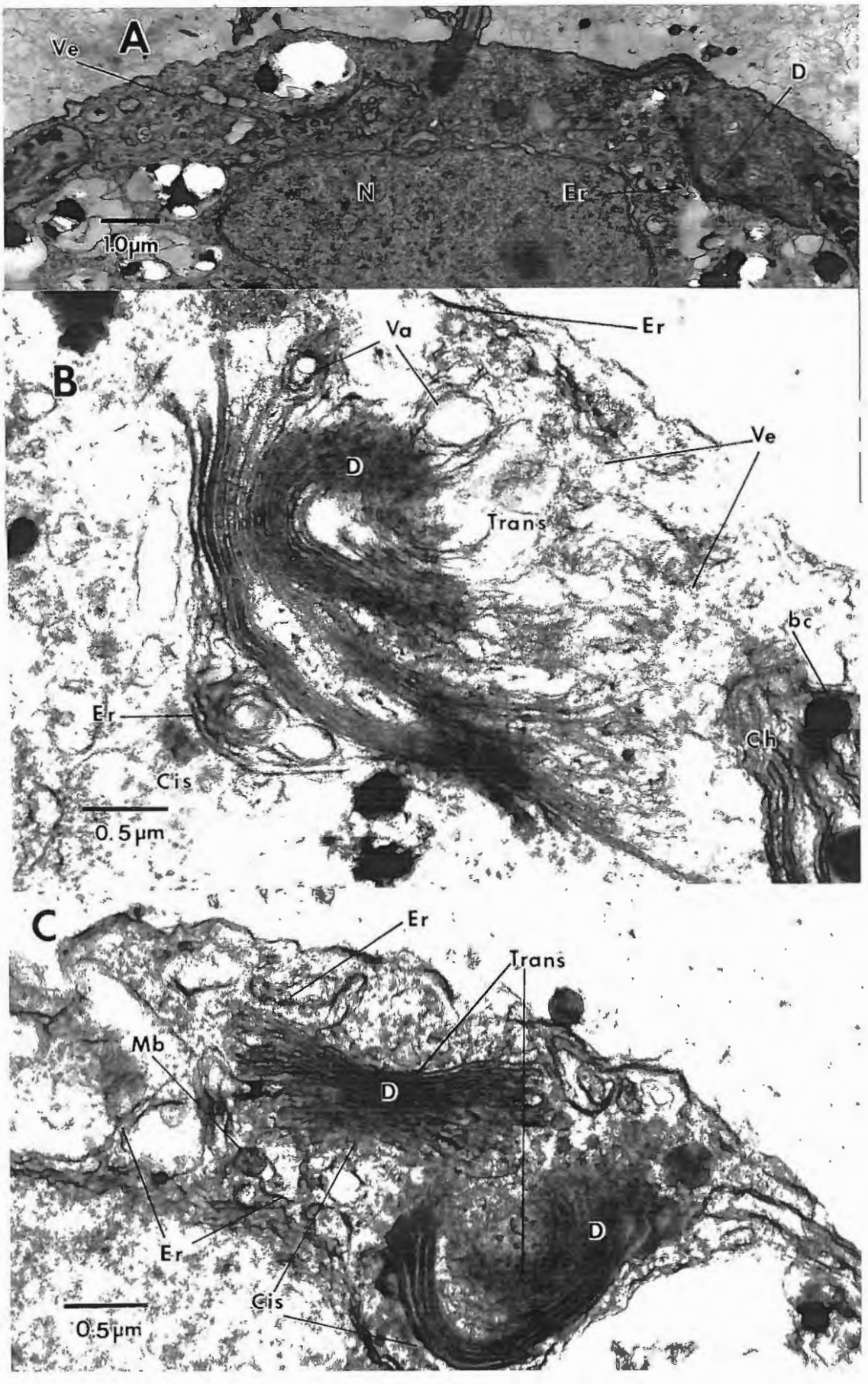
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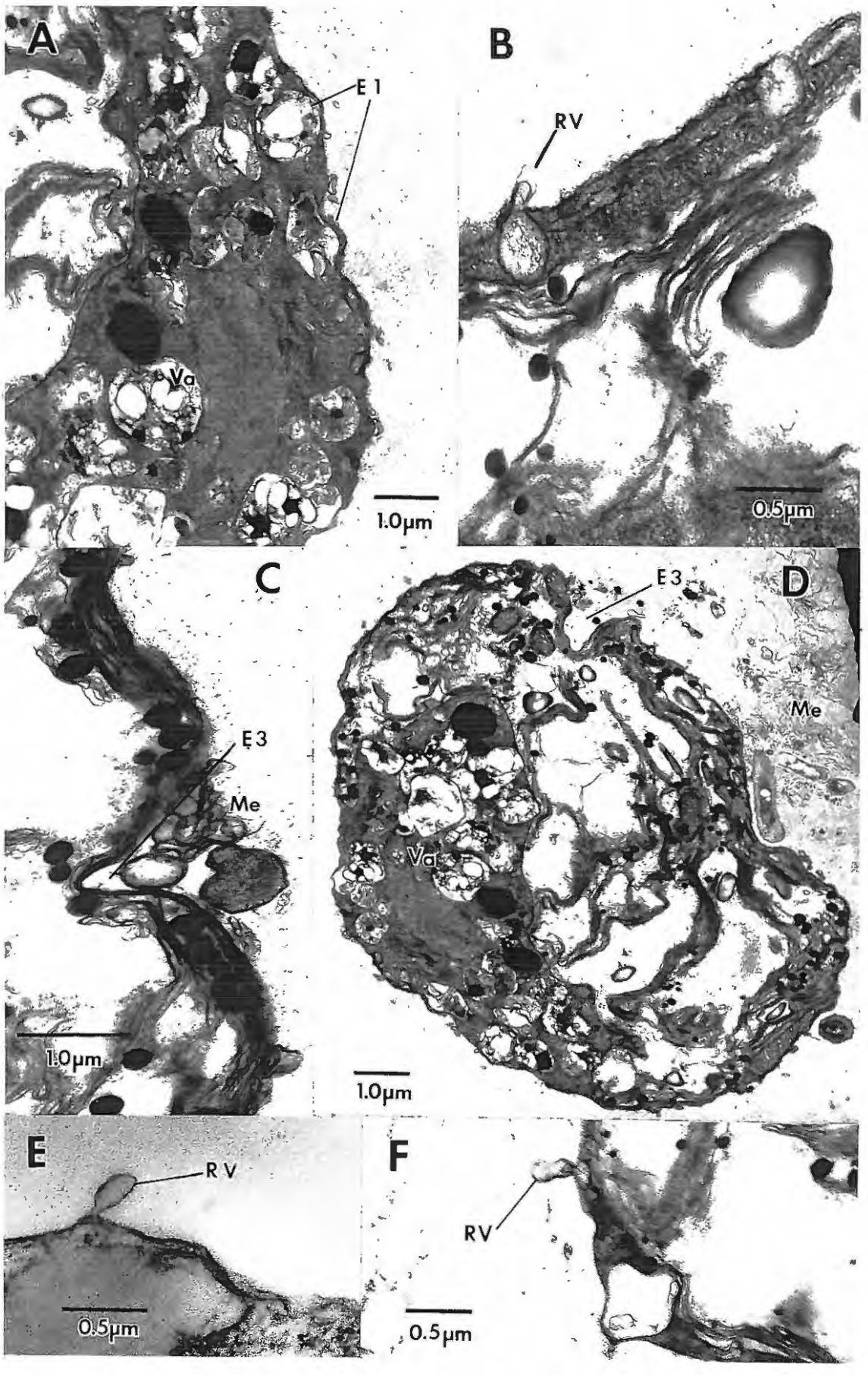
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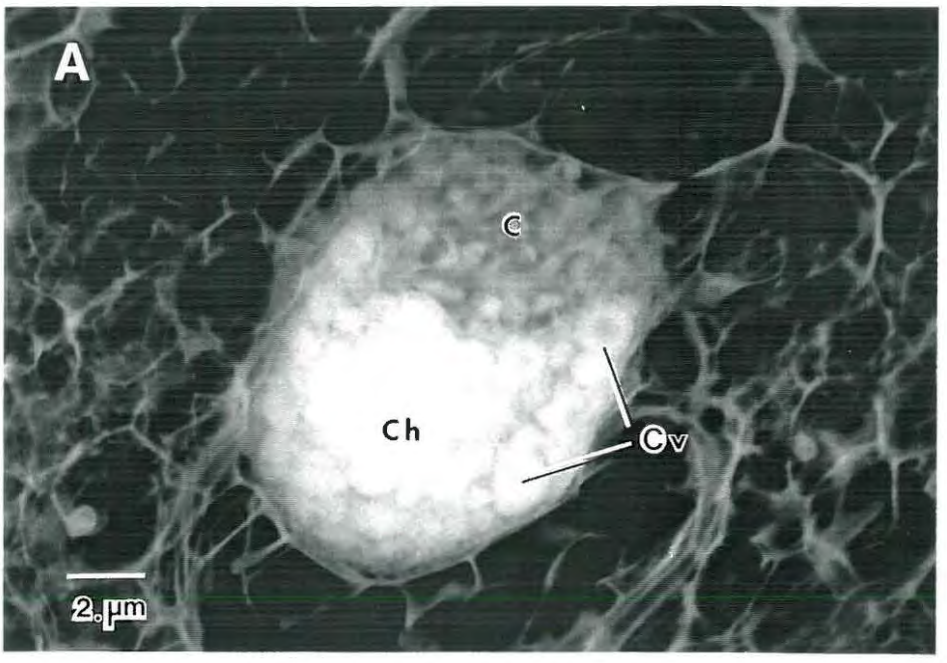
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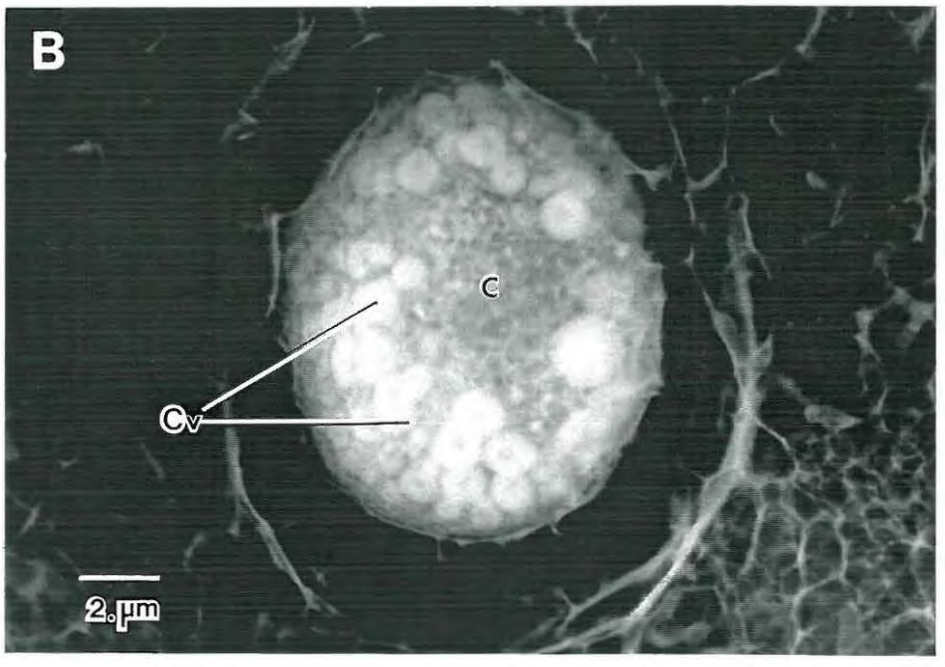
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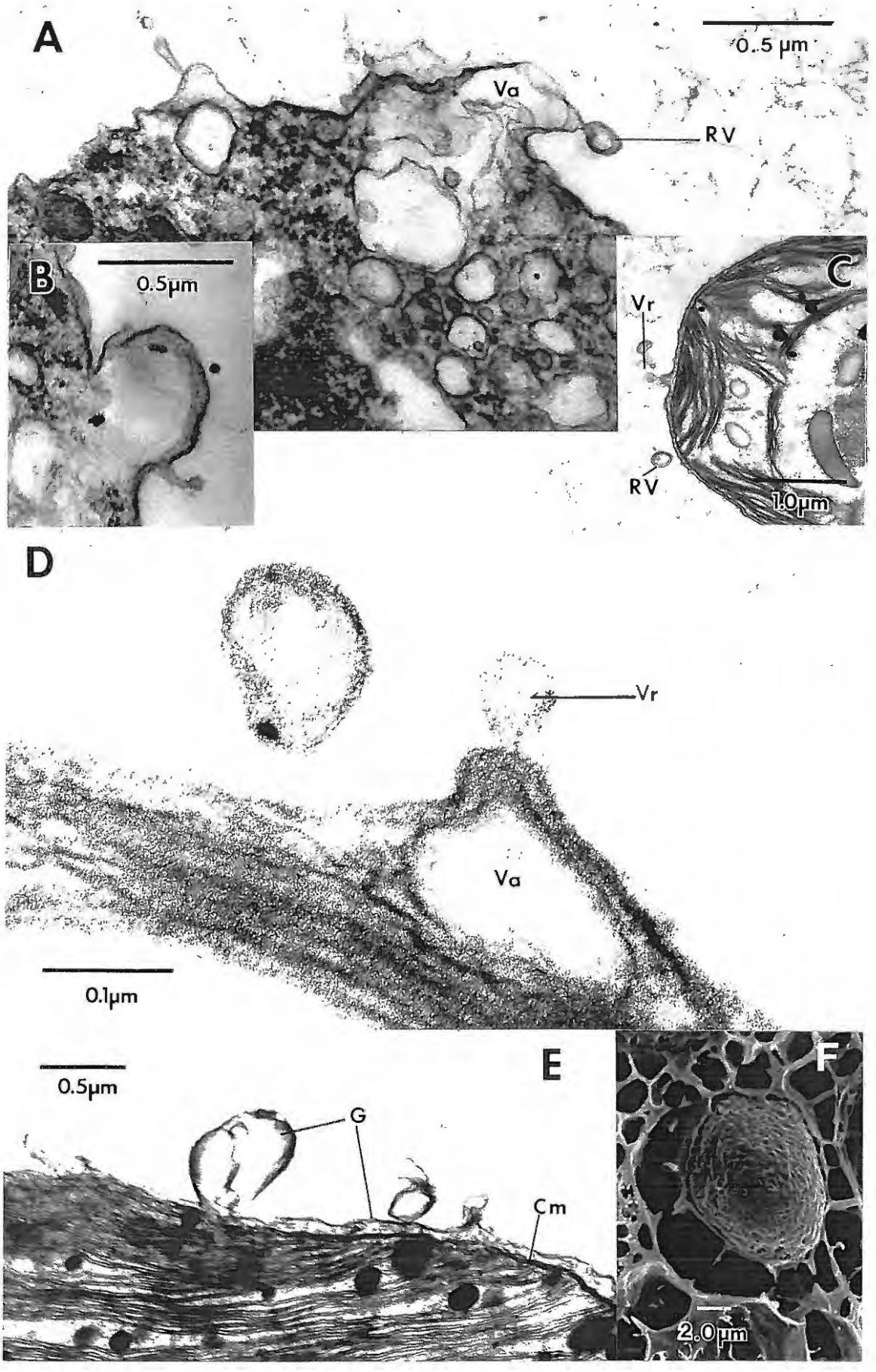
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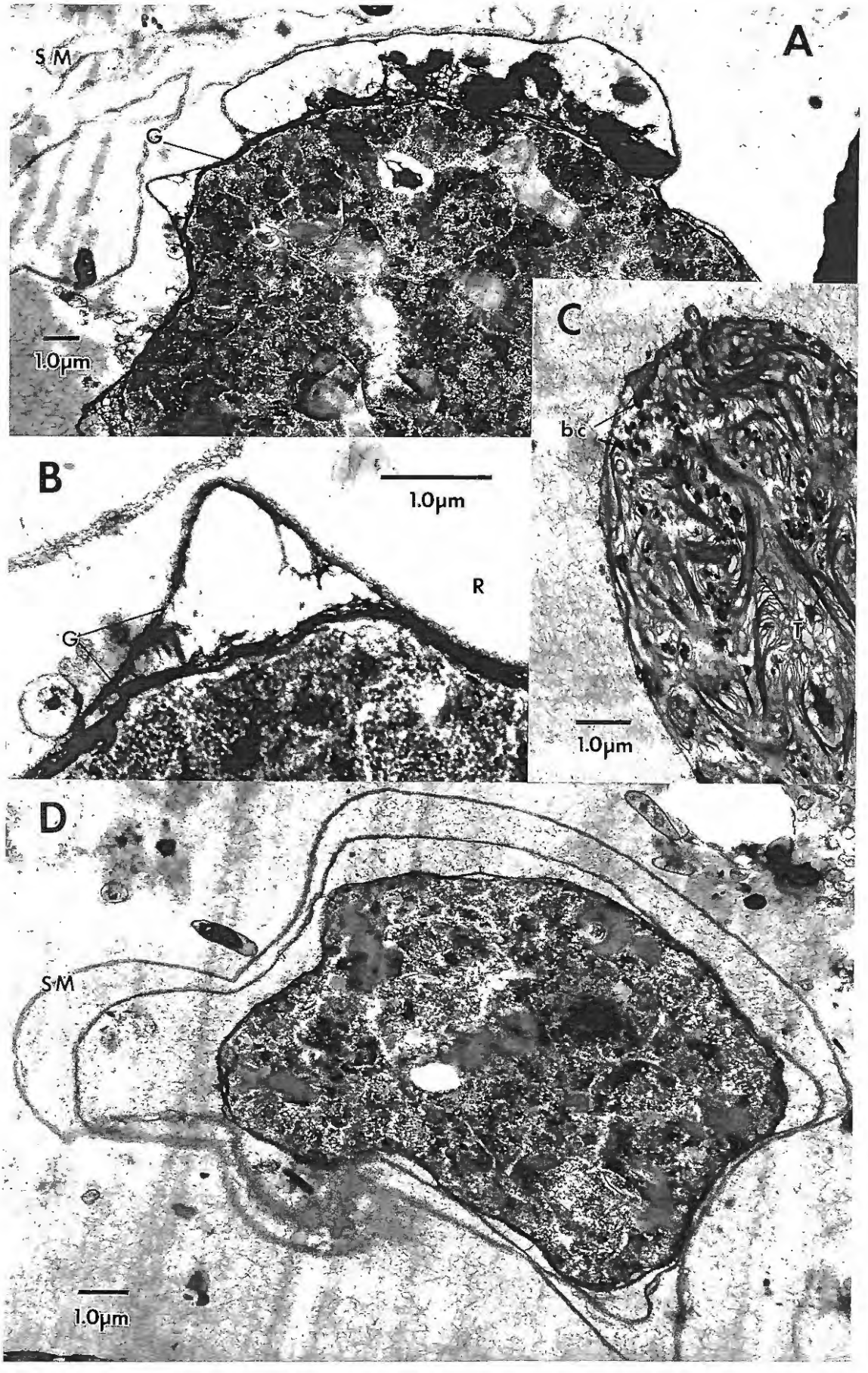
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CHAPTER 7

STRESS, THE MANIPULATION OF *DUNALIELLA* AND MULTISTAGE PROCESSING

Summary.

A comparison of various options for the stress induction of β-carotene and glycerol accumulation by *D.salina* demonstrated the practical advantages of a combined nitrogen/salinity stress, applied under the conditions of low light, that occur with high cell densities and in partially turbid media. To manipulate stress factors for commercial algal metabolite production, a multistage production process has been proposed where cell growth and product accumulation can be optimized in separate unit operations. A provisional benefit estimate for this approach indicates a potential four-fold yield enhancement compared to conventional production methods.

7.1. INTRODUCTION.

The application of physiological and environmental stresses as manipulative factors in managing cultures of micro-organisms and for the induction of metabolite production are well described techniques in biotechnology. This generalization applies equally to *Dunaliella* and a considerable body of literature has emerged dealing with the stress responses of this organism and the induction of metabolite production (Lerche,1937;Mil'ko,1963; Mironyuk&Einor,1968;Aasen *et al.*,1969;Pace *et al.*,1977;Ben-Amotz&Avron,1973,1980,1983,1989a;Ben-Amotz *et al.*,1982;Semenenko&Abdullaev,1980;Loeblich,1982;Borowitzka *et al.* 1984,1986,1990;Sadka *et al.*,1989,1991;Lers,1990;Cowan&Rose,1991). Glycerol is produced as an osmoticant in response to salinity stress and can accumulate to 40% cell dry weight (Ben-Amotz,1980) which enables *D.salina* to tolerate the highest halostress of any known eukaryote (Brock,1975;Borowitzka&Borowitzka,1988b). A range of stress factors including high light intensity, salinity and temperature, low levels of nitrogen, phosphates and iron and heavy metal contamination have all been associated with the exceptional production of large quantities of β-carotene by certain strains of *Dunaliella salina* - specifically the *bardawil* variety (Ben-Amotz&Avron,1980). Accumulation of β-carotene up to 10%-13% (Ben-Amotz&Avron,1989a) or 14% of cell dry weight (Borowitzka *et al.*,1986) has been reported. Features of the β-carotene response will be discussed below.

7.1.1. Light Stress.

Mil'ko (1963) had drawn attention to the role of light intensity in the induction of β-carotene synthesis. Ben-Amotz and Avron (1983) have shown that the extent of β-carotene accumulation is a direct function of the integral amount of light to which the algae are exposed during a division cycle. The maximum accumulation

is observed when cells are exposed to high light intensity and simultaneously subjected to growth-limiting conditions. Ben Amotz *et al.* (1989b) claim a major photoprotective function for β-carotene as a screen absorbing excess irradiance and limiting photoinhibition. An argument for a chlorophyll stabilizing effect, with β-carotene quenching singlet oxygen produced under high light intensities, had previously been advanced by Foote (1976), Brown and Borowitzka (1979). The location of β-carotene globules in the interthylakoid space of the chloroplast pointed out by Ben-Amotz *et al.* (1982) contradicted a previous report by Aasen *et al.* (1969), that in *Dunaliella* accumulation was extraplastidic.

A characteristic feature of the stress response is a substantial elevation of the carotene:chlorophyll ratio and Smith *et al.* (1990) have shown that high light intensity promotes a smaller chlorophyll antenna with the accumulation of photochemically inactive units of photosystem II. Nelis and De Leenheer (1991) note previous reports of carotenoid production in a wide range of micro-organisms, from bacteria to higher plants, and conclude that in most cases β-carotene biosynthesis has been found to exhibit a partial or complete dependence on light. Lers *et al.* (1990) have reported the kinetics of photoinduction of β-carotene in *D.salina*, stressed in a light range from 27-1650μmol.m^{-2}.sec^{-1}. While they found that the effect of light intensity on β-carotene production is independent of growth rate, high light intensity is, in itself, a stress factor, and generation times doubled for cells stressed to 1650μmol.m^{-2}.sec^{-1}.

Ben-Amotz and Avron (1983) and Moulton *et al.* (1987a) conclude that the light shading, photoprotective function for the massive accumulation of β-carotene accounts for the dominance of high-producing strains over other halophilic forms (e.g. *D.viridis* and non-accumulating forms of *D.salina*) in natural environments such as salt lakes.

7.1.2. Nutrient Stress.

The general concept of metabolite induction by nitrogen-stress in the micro-algae has been known for a long time and Richmond (1986d) quotes the accumulation of lipids in diatom cultures in response to nitrogen limitation, reported by Harder and von Witsch in 1942. The nitrogen-stress effect has been demonstrated in numerous algae, including *Haematococcus* (Droop,1961), *Nannochloropsis* (Sukenik *et al.*,1990) and *Porphyridium* (Arad *et al.*,1986).

Lerche (1937) had suggested that the red colour in *Dunaliella* was due to a deficiency in nitrogen and phosphate and Mil'ko (1963) and Semenenko and Abdullayev (1980) have demonstrated this effect. Ben-Amotz (1987) and Lers *et al.* (1990) have showed a similar effect for sulphate deficiency. Borowitzka and Borowitzka (1988b) have identified nitrogen limitation as the major causal factor in the synthesis and accumulation of carotenoid pigments among the Chlorophyceae, but point out that any condition leading to cessation of growth will initiate the production of carotenoids. They suggest that carotenoid accumulation may not be the result of the limitation of nutrients *per se*, but rather the effect that stress has on the metabolism of protein and nitrogen. A substantial

argument has been advanced by Hellebust (1965,1985), Ignatiades and Fogg (1973) and others that the carotenoids and other similar metabolites produced, and in some cases excreted, by the micro-algae, arise as secondary metabolites. They serve as a carbon sink for the excess photosynthate produced when cell growth slows down at the onset of stationary phase.

7.1.3. Salt Stress.

Salt stress was also recognised early on as an important factor in the induction of carotenogenesis in *Dunaliella* (Labbe,1921) and has been investigated by Mil'ko (1963), Ben-Amotz and Avron (1980) and Borowitzka *et al.* (1984). An inverse relationship between salinity and the specific growth rate of *D.salina* has been demonstrated by Ben-Amotz *et al.* (1982) and Al-Hassan *et al.* (1987). Mil'ko (1963) has reported a reduced carotene accumulation effect for salt stress compared to nitrogen and phosphate deficiency stresses.

Borowitzka *et al.* (1990) have studied the kinetics of β-carotene induction in hypersalinity-shocked *Dunaliella*. They report an initial lag in accumulation which is consistent with transition to a stationary phase growth pattern, followed after about two days by the rapid accumulation of pigment. They conclude, however, that the mechanism for this response remains elusive. Arguments that the physiological responses reported above serve a function to protect the alga against the potentially damaging effects of stress, raises the possibility that they might be hormonally mediated (Hirsch *et al.*,1989;Tietz *et al.*,1989; Zeevaart&Creelman,1988; Bradley,1991; Cowan&Rose,1991;Evans&Trewavas,1991).

7.1.4. Stress Synergism.

The value of a synergistic interaction of different stress factors has also been noted. Mil'ko (1963) reported a synergistic enhancement of β-carotene accumulation for a combined light and temperature stress. Borowitzka *et al.* (1986) report that *Dunaliella salina* under nutrient limitation plus high salt (25%) produces up to 14% carotenoids as cell dry weight, of which β-carotene comprises 95%. On the other hand, nutrient limitation with 10% salt results in a yield of only 3.9% carotenoids as cell dry weight, of which just 30% is β-carotene. Ben-Amotz and Avron (1990) have demonstrated a similar synergism for light and salinity and report that 4M NaCl plus 550kErgs.cm^{-1}.sec^{-1} produces the highest β-carotene:chlorophyll ratio of 13 (i.e. 12%-13% β-carotene as cell dry weight).

Ben-Amotz and Avron (1983) have proposed separate stress mechanisms for β-carotene accumulation: the response to high light and the response to those environmental stresses which induce a growth limitation such as salt, nutrient deficiency or temperature. A demonstration of two clearly separate stress induction effects have been reported by Lers *et al.* (1990). Sudden exposure to a high light stress from 27 to 1650μmol.m^{-2}.sec^{-1} produces an immediate response. This phase lasts about 24 hours with an accumulation of approximately 20pg.cell^{-1} β-carotene. It is followed by a second phase of accumulation which is comparable, in both timing and rate, with the onset of stationary growth that occurs about two days after the application of a nutritional

stress. A sulphate deficiency stress was used in this study. Borowitzka *et al.* (1990) also confirmed a two day lag in β-carotene production after the application of a hypersalinity-shock from 15% to 30% NaCl. Attention is drawn to the photoprotective function of the initial response to light, but the reason for the second response at stationary phase remains obscure.

7.1.5. Conventional Processing.

The practical implications of tbe physiological responses of this organism to stress provides the basic dilemma of the large scale commercial process for β-carotene production using *Dunaliella* - accumulation is generally greatest when the growth rate is least (Borowitzka *et al.*,1990). Ben-Amotz *et al.* (1982) demonstrated an inverse relationship between specific growth rate and the β-carotene content of *D.salina*. Ben-Amotz and Avron (1989a) have concluded that "...*Dunaliella* represents a special case in optimizing productivity. The aim is not to increase the productivity of algal biomass but to maximise the production of β-carotene - even at the expense of algal biomass". These conflicting requirements result in the inherent low productivity of large-scale outdoor pond production systems. An "averaging" approach is used where cells are grown at salinities (2.5-3.5M NaCl) sufficiently elevated to overcome the problems of contamination and competition by non-producing species of *Dunaliella*, but way above the level required for optimum growth rates (1.5M NaCl). The nitrogen levels required for adequate cell growth and the high populations required to prevent contamination by other *Dunaliella* species (5-10mM KNO_3) in turn limits the effect of stress-induced synthesis of high levels of β-carotene, which requires that nitrogen levels fall below 1mM KNO_3 (Ben-Amotz&Avron,1983). Intricate modelling is used to derive an optimum within these conditions (Moulton *et al.*,1987a;Guterman, *et al.*,1990).

As a result, practical yields achieved in the long term are about 10%-25% of the theoretical potential of the system (Ben-Amotz&Avron,1990). Care must, nevertheless, be exercised in extrapolation from laboratory-acquired experimental data. The reduction in yields, of algal systems transferred from laboratory to outdoor conditions is to be anticipated and has been well documented (Goldman,1979;Moulton *et al.*,1987a).

Glycerol production by *Dunaliella*, in direct contrast to the factors determining β-carotene accumulation, is dependant on maximum growth rate and maximum cell production (Ben-Amotz&Avron,1980;Chen&Chi,1981).

7.1.6. Multistage Processing.

To address the problem of low β-carotene yields, the idea of a two stage process has been proposed by a number of authors, where the biomass production and product accumulation functions would be unlinked and optimised in separate unit operations (Mil'ko,1963;Massyuk&Abdullaev,1969;Borowitzka *et al.*,1984). In this approach growth would be optimized under conditions favouring maximum cell production (high nitrogen and low salinity), followed by an exposure to physiological stresses where metabolite production, both glycerol and β-carotene would be maximised. Up to the present time only the application of salinity stress appears to have

been considered (Chen&Chi,1981;Moulton *et al.*,1987a;Borowitzka&Borowitzka,1988b) and this evaluation has been largely based on theoretical considerations.

Moulton *et al.* (1987a) report that they had dismissed, at an early stage of their development programme at Hutt Lagoon, Western Australia, a system based on the relatively fast growth of *D.salina* at a low salinity (approximately 150g.L^{-1}) followed by increased salinity (approximately 250-300g.L^{-1}) to produce higher concentrations of β-carotene. They comment that their further experience had confirmed the infeasibility of this approach. Moulton *et al.* (1987b) have demonstrated, in species interaction studies, that the use of lowered salinities to increase cell production is unfavourable given the loss of control over competition and predation. This assessment appears to be based on the idea that the primary advantages of the multistage approach would be found in enhanced cell production followed by a salt stress-induced accumulation of β-carotene in the presence of the original growth medium. Neither assumption turns out to be supportable, as will be discussed below.

Despite the theoretical advantages offered by the multistage approach, little or no practical development work has been undertaken, and with the decline of interest in glycerol production, the idea has been discarded as a model for commercially-scaled *Dunaliella* cultivation. There are, nevertheless, important incentives for the further evaluation of the idea given the marginal profitability of algal biotechnology production processes and inherently low yields in a high production-cost operation.

In practice the problems of marginal profitability can be addressed by either increasing productivity and/or reducing costs. Where a critical analysis of a process is undertaken to indicate where further research development would be rewarding, Borowitzka and Borowitzka (1989b) warn that this should be aimed at a substantial sustained advantage rather than merely marginal improvements to the economics of the process.

The reduction of costs to be anticipated with the use of certain saline effluents as alternative sources of media and the credits associated with the use of effluent-treatment ponding facilities have already been noted. Appropriate steps to increase metabolite yield from the system would provide another obvious research direction. This could involve an enhancement of cell numbers and/or β-carotene content per cell.

It will be argued here that, given the development of appropriate cell separation technology effecting algal biomass transfer from one medium to another, both cheaply and without impairment of cell viability (see Chapter 9), and more precise control over the metabolite stress induction process (see Chapter 8), that the multistage approach to algal production could be incorporated into a process design to improve approximation of the full theoretical yield-potential of the alga.

While the process discussed so far essentially involves only two stages, and will be referred to as such where appropriate, the potential for incorporating other steps, identified in chapter 10, led to the use of the "multistage" term as a more comprehensive descriptive title.

7.1.7. Costing.

The objective of increasing productivity in terms of final product yield cannot be viewed in isolation from the influence of the costs of production. Borowitzka (1991) has estimated the cost of producing dried *Dunaliella* biomass to be about Aus \$20 000.ton^{-1} including packaging and marketing costs. This cost rises to about \$30 000.ton^{-1} where high-cost harvesting such as centrifugation is used. This may be close to the current market value of the dried algal product (containing approximately 5% β-carotene) used for animal feed applications. The estimate does not include pharmaceutical grade extracts. This marginal nature of the current methods of algal β-carotene production provides a substantial incentive for technological innovation.

Oswald (1988b) has generalised costings for the large-scale open pond algal culture enterprise. Harvesting and handling accounts for 33% and cost of medium alone accounts for between 25%-33% of total production costs. Pond construction, depending on the type used, can account for over 70% of capital investment. The balance accounts for all other costs including capital structures, amortization, interest, labour etc. Tapie and Barnard (1988) have provided costs for tubular photobioreactor systems with centrifugation accounting for about 20% and media preparation about 14% of operating costs. These somewhat lower values are probably due to the high total costs of tubular systems. De Pauw and Persoone (1988) have recorded a 20% contribution to production costs by the nutrient requirement in the generation of artificial phytoplankton blooms to be used for aquaculture feeds. Total costs of biomass production, which does not include mechanical harvesting, may range from US\$4 to 20.Kg^{-1} dry weight with enclosed and indoor production rising to US\$160.Kg^{-1} dry weight.

Moulton *et al.* (1987a) have used computer-based bioeconomic modelling of the complex interrelationship of the variables in β-carotene production to identify the cost sensitivity of the various features of the process and to assess overall feasibility. They have shown that the relationship that exists between the high variable costs of harvesting and extraction (which are associated with the volume to be processed), and the relatively lower variable costs of growing the algae, makes the elevation of the β-carotene level in the cell tbe most sensitive cost area to manipulate. This also governs the optimum point in a culture at which harvesting should commence and is a compromise between maximum cell number and β-carotene yield. These authors have determined this point as 85% of the β-carotene maximum. They conclude that if the harvest level of β-carotene can be raised in any way other than by waiting for further growth, the overall production cost will be reduced. Thus those factors which increase productivity by increasing carrying capacity are more beneficial than those that increase growth rate. Any method that increases cell density at harvest is also likely to be beneficial.

Borowitzka (1991) has produced a cost sensitivity analysis which confirms earlier findings. This indicates that while a 10% reduction in costs can be anticipated from optimizing cell densities, increasing the cellular concentration of β-carotene is sensitive up to a 50% reduction in costs.

Given that the currently accepted "averaging" approach to production does not enable an even close approximation of the theoretical yield potential of the system, the multistage approach may present the only way of meeting the productivity optimization objectives required to address the problem of marginal profitability.

7.1.8. Effluent-Grown *D.salina*.

While the advantages of multistage processing could offer theoretical yield enhancement in a defined medium production process, the accumulation of β-carotene by *D.salina* in nitrogenous effluents would, however, require as a prerequisite, the particular application of the concept. The satisfactory induction of β-carotene synthesis and its accumulation to high cellular levels has been shown to be dependant on a number of stress factors which can be the subject of complex interrelationships, but underlying these is the requirement for an absence of excess nutritional nitrogen. It is implicit in the results already reported (see figures 3.5,3.6,3.10) that the removal of cell biomass from the growth medium and its transfer to nitrogen-free conditions would be a prerequisite for β-carotene production by *D.salina* in an effluent such as Hide Soak Liquor. It is apparent that without this change of medium, β-carotene production would not be feasible within the tannery effluent treatment concept that has been outlined.

Two approaches have been identified that could be followed in implementing a multistage system for handling cells that have been produced in a HROP treating tannery effluent:

1. Active Multistage

   Cells could be actively removed from the growth medium by the application of appropriate cell separation technology and the cell concentrates transferred, in a viable form, to a nitrogen-deficient (optionally saline) stress medium, where product induction and accumulation could occur.

2. Passive Multistage

   The HROP-treated effluent containing the algal biomass could be discharged to an evaporation ponding cascade, such as that described in Chapter 2, where a passive process of gradually increasing stresses would be applied. Salinity levels would rise and excess nitrogen would be consumed with cells harvested from the brine concentrate at the end of the process.

Clearly, to proceed with this line of reasoning, a substantial re-evaluation of the multistage concept would be required. The question to be answered is whether the potential of the multistage approach has possibly been overlooked, given that the focus of the initial evaluations had fallen primarily on hypersalinity-shock aspects

rather than the application of nutritional deficiency or other stresses. Also the practical implications of repeated water transfers on a large-scale, the effective manipulation and control of the stresses and the appropriate cell separation technology that will be required present substantial design and operational problems. This latter aspect will be dealt with in Chapter 9 but the issue to be clarified first is whether there is sufficient information presently available in the literature to enable a provisional estimate of feasibility. While any one of the numerous aspects of multistage processing could form a major study in itself, the remainder of this investigation will be focused on acquiring sufficient information to answer the aforementioned questions of provisional feasibility.

7.2. RESEARCH OBJECTIVES.

The literature reviewed contains numerous references to the effects of, and the final β-carotene yields produced by individual stresses or combinations of these acting synergistically. Most reported results reflect absolute, time-unrelated values for β-carotene production. Rate data for individual stresses indicating the magnitude of production per unit time have been published, but it appears that a comprehensive comparison of the rates for the different stress factors has not yet been undertaken. Clearly such a comparative evaluation is required to enable an assessment of the relative utility of the various stress options available for the successful manipulation of *Dunaliella* cultures, and that could be considered in the design of a multistage process.

The experimental programme outlined below was not designed to undertake such a comprehensive comparative evaluation but rather to fill in gaps apparent in the literature and supplement the information already available.

The following experimental objectives were identified:

1. To compare the effects on biomass yield of two forms of stress, nitrogen and salinity, which can be applied to *Dunaliella*;
2. To confirm the previously reported synergistic effects of salinity and nitrogen-stress and to compare the use of these two stresses on β-carotene production in *D.salina* under conditions of reduced light, as would be anticipated in turbid effluent-sourced media;
3. To compare the two practical options (Active and Passive Multistage) for applying stress to *D.salina* cultures, produced during the HROP treatment of saline organic effluent.

7.3. MATERIALS AND METHODS.

Cells were grown in ASW and Hide Soak Liquor formulated media and all analyses performed as previously described.

The nitrate exhaustion study was set up by transferring a washed culture of effluent-grown *D.salina* into both nitrogen-deficient (N^-) and standard ASW (N^+) media at a cell concentration of $20x10^4.mL^{-1}$. Cell counts, β-carotene and nitrate levels were recorded over a 60 day period.

The Active Multistage evaluation study was set up by transferring washed Hide Soak Effluent-grown cells and ASW-grown controls to nitrogen-deficient (N^-) and standard ASW (N^+) media at cell concentrations of $20x10^4.mL^{-1}$. β-carotene levels were recorded over a 15 day period.

Different units of light measurement encountered in the literature comparison of β-carotene production rates were standardized to $\mu mol.m^{-2}.sec^{-1}$. The conversion of cellular levels of β-carotene in picograms to yield values expressed as a percentage of algal biomass dry weight, was based on average cellular values provided by Ben-Amotz *et al.* (1982).

7.4. RESULTS.

Both salinity and nitrogen-stress have a marked impact on the growth rate (μ) and productivity (cell number) of *D.salina*. Figure 7.1 shows that in a salinity-stressed environment (at the 3M NaCl upper limit that is normally maintained in "averaged" open ponds) there is a 45% lower growth rate than at 1.5M NaCl. If a nitrogen-stress is imposed at the same time then productivity can fall to a 65% reduction in growth rate. Expressed differently, where cells are grown at 3M NaCl to prevent predation and competition effects, the further imposition of a nitrogen-stress can result in an additional 37% reduction in growth rate.

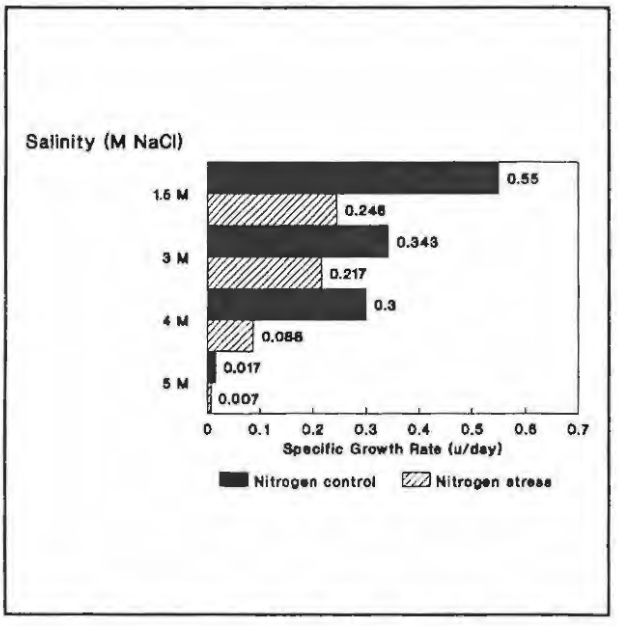


Figure 7.1 The combined effects of salinity and nitrogen-stress on the growth rate (μ) of *D.salina* in ASW medium.

As could be anticipated the reduction in growth rate is reflected by an equivalent fall in cell productivity. Figure 7.2 shows a reduction in cell yield of 46% with an increase in salinity from 1.5M to 3M NaCl measured at the 10 day retention time. The further elevation of salinity to 4M NaCl results in a 70% drop in cell yield as compared to the yield at 1.5M.

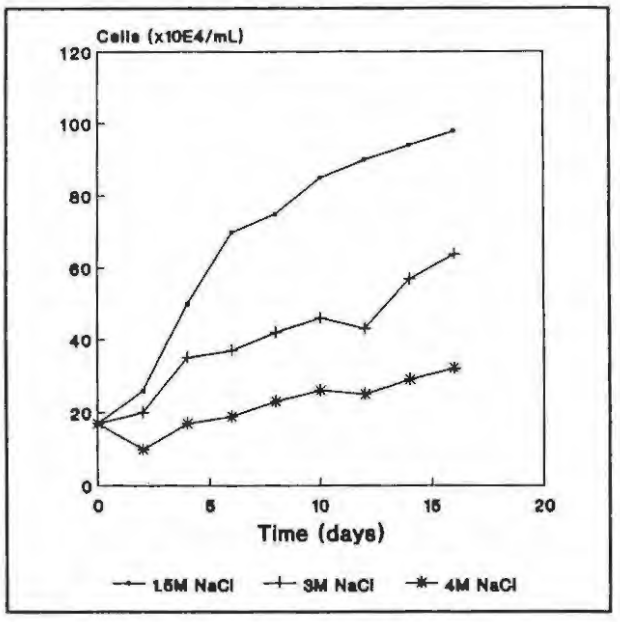


Figure 7.2 The effect of salinity stress on the cell productivity of *D.salina* grown in ASW medium, measured as a function of cell number.

It was found in preliminary experiments of cell exposure to high light intensities (results not shown) that the massive β-carotene production response obscured the observation of nitrogen and salinity interaction effects. Consequently it was decided to conduct the subsequent investigation at reduced light intensities where the nitrogen and salinity effects could be observed, but at the same time without the quenching of β-carotene induction caused by cell shading, and thus rendering the system insensitive. The results recorded in figure 7.3 show that increasing cell numbers have a substantial effect on reducing β-carotene production (measured as β-carotene:chlorophyll ratio) and that the most sensitive result would be expected at cell densities of about $20x10^4.mL^{-1}$ where cultures were grown under the somewhat arbitrary conditions described above. This cell density was used in the subsequent stress experiments reported below.

Also apparent from figure 7.3 is the marked combined effects of both nitrogen-stress and increased exposure to light on cellular β-carotene production. At the highest light intensity, produced by adjusting cell number, the simultaneous imposition of nitrogen-stress produced a six-fold increase in the β-carotene:chlorophyll ratio. It is also apparent that at the low light intensity conditions that pertained in this experiment, virtually no effect on β-carotene production is observable without the imposition of a nitrogen-stress.

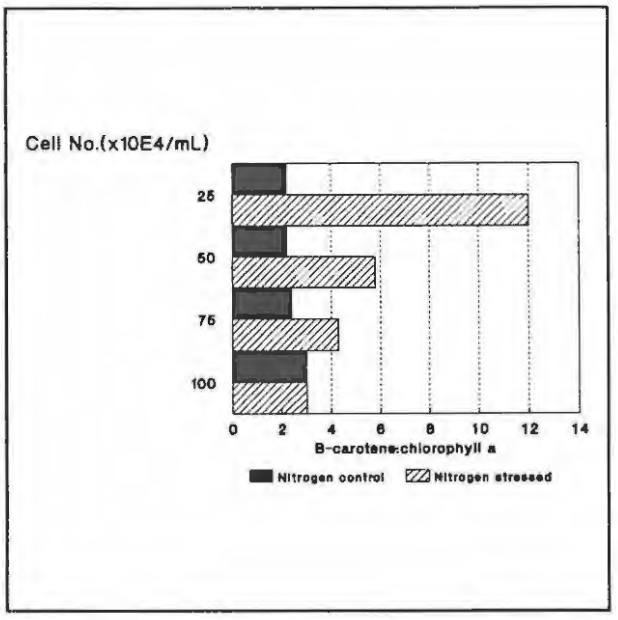


Figure 7.3 The combined effects of light intensity (cell density shading) and nitrogen-stress on β-carotene production (β-carotene:chlorophyll ratio).

The interrelationship of salinity and nitrogen-stress effects on β-carotene production showed opposite results when comparing yields as measured per cell and as a percentage of cell dry weight (figures 7.4,7.5). Additional cell growth occurred at the lower salinity values, and while the cellular levels of β-carotene were lower, the cell mass increase accounts for the enhanced production of β-carotene. This masks the observation of higher cellular levels of β-carotene produced at elevated salinities where cell mass measurements are compared. Where dense climax cultures are to be used the results indicate that a combination of salinity and nitrogen-stress will result in the greatest accumulation of β-carotene per cell. However, a comparison of β-carotene:chlorophyll ratios (figure 7.6) indicates that the highest ratios are, in fact, achieved under conditions of nitrogen-stress at lower salinities. Figure 7.4 and 7.5 also show the comparatively insignificant advantage in imposing a salinity stress, in excess of the approximately 3M NaCl under which the cells would be grown, in the absence of a simultaneous nitrogen-stress.

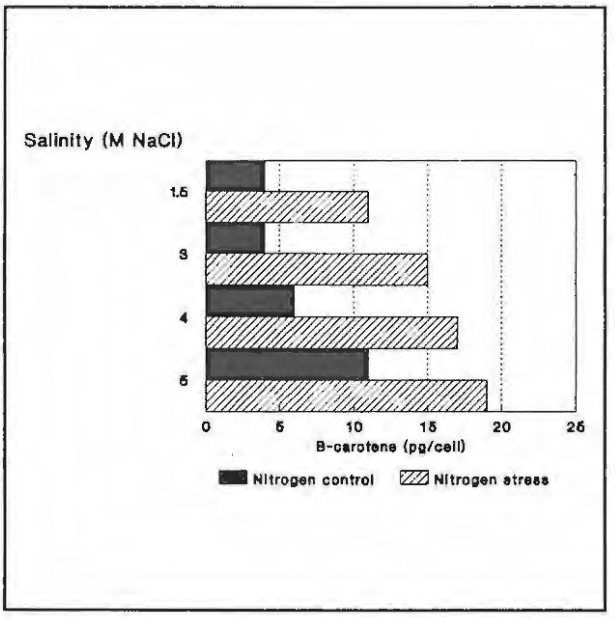


Figure 7.4 Combined stress effects of salinity and nitrogen on β-carotene production in *D.salina* grown in ASW medium, measured as yield per cell.

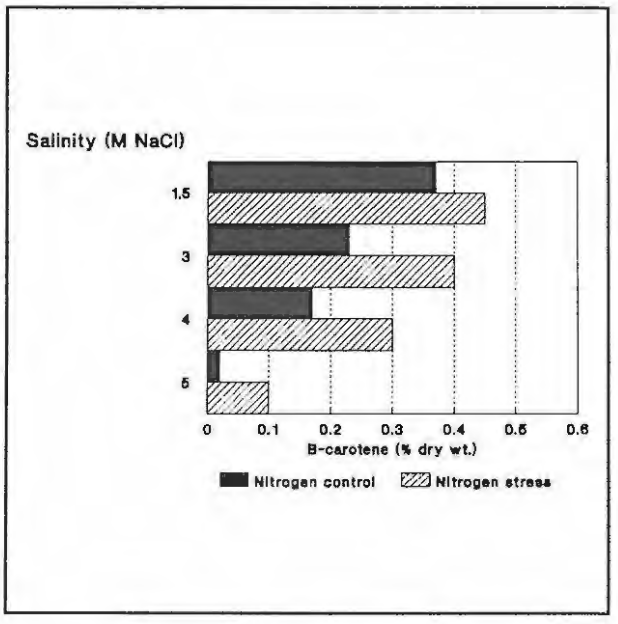


Figure 7.5 Combined stress effects of salinity and nitrogen on β-carotene production in *D.salina* grown in ASW medium measured as a percentage of cell dry weight.

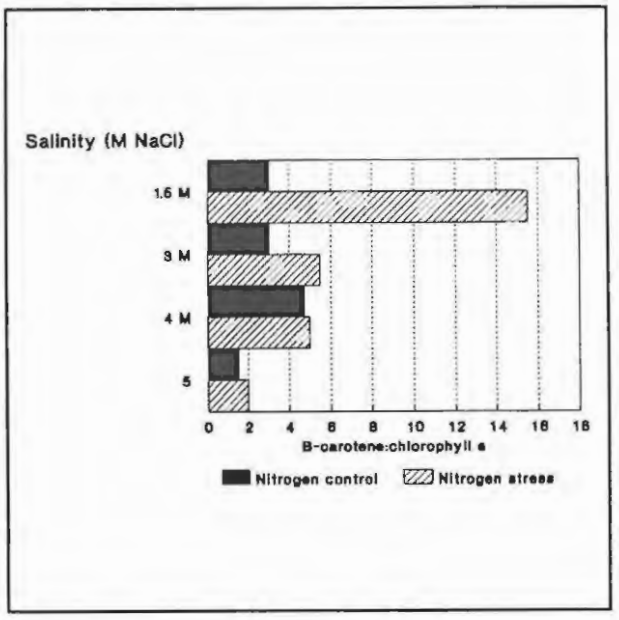


Figure 7.6a Combined stress effects of salinity and nitrogen on β-carotene production by *D.salina* measured as the β-carotene:chlorophyll ratio.

The effects on β-carotene production of imposing nitrogen-stresses on effluent-grown *D.salina* cells has already been documented (see figures 3.5,3.6,3.10). The results are essentially similar to those reported above for growth in the semi-defined ASW medium with the exception of the nitrogen carry-over effect observed in effluent-grown cells. The comparison, however, involved the application and removal of only nitrate as the sole source of nitrogen in the ASW-grown cells, while in the effluent-grown cells the nitrogen source was mixed including both organic and inorganic nitrogen expressed as COD, PV, protein, ammonia and nitrates. To allow for a more valid comparison of the two forms in which the cells were grown, a correlation was made between cellular β-carotene production and nitrate levels, in cells derived from different strengths of effluent and transferred to nitrogen-deficient stress media. Figure 7.7 records the inverse correlation between the nitrate level of the effluent growth medium and final β-carotene production. The results further confirm the observation that the β-carotene production response is similar for cells grown in both types of media.

Figure 7.6b Carotenoid productivity indicated as colour change with the application of salinity stress alone at 1.5, 2.5, 3.5 and 4.5M NaCl (top) compared with a combined nitrogen-deficiency stress applied over the same salinity range (bottom).

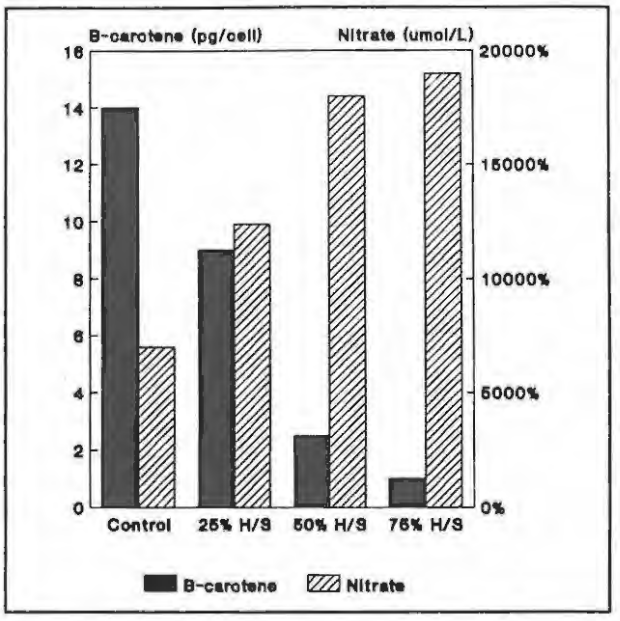


Figure 7.7 β-carotene production by *D.salina* as a function of nitrate levels in Hide Soak Liquor derived growth media.

Figures 7.8 and 7.9 record the results of two possible practical methods identified above which could be used to apply stress to the alga viz: the active and the passive multistage approaches. Allowing for a natural removal of nitrogen (measured as nitrate) by cell growth, using effluent-grown cells transferred to a nitrogen-containing ASW medium, an inverse relationship is demonstrated. The β-carotene levels start to rise only when nitrate has fallen to a certain critical level due to its consumption. This level appears, from these results, to be in the vicinity of about 100 μmol.L^{-1} nitrate (figure 7.8). Figure 7.9 shows the steady increase in cell number over the 60 days of the experiment when compared to the nitrogen-deficient control. This serves to confirm the assumption that nitrate removal was effected by cell consumption. Clearly light was not a limiting factor.

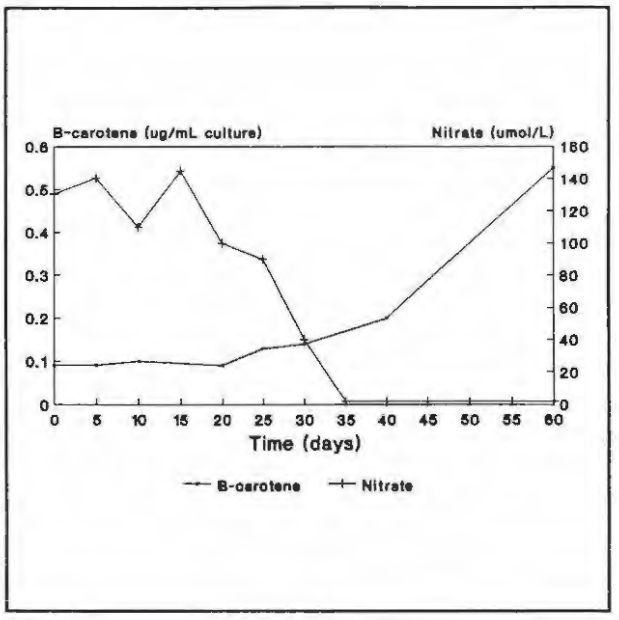


Figure 7.8 β-carotene production and nitrate consumption in *D.salina* cells grown in Hide Soak Liquor. Cells were washed and transferred to nitrogen sufficient ASW medium and incubated for 60 days.

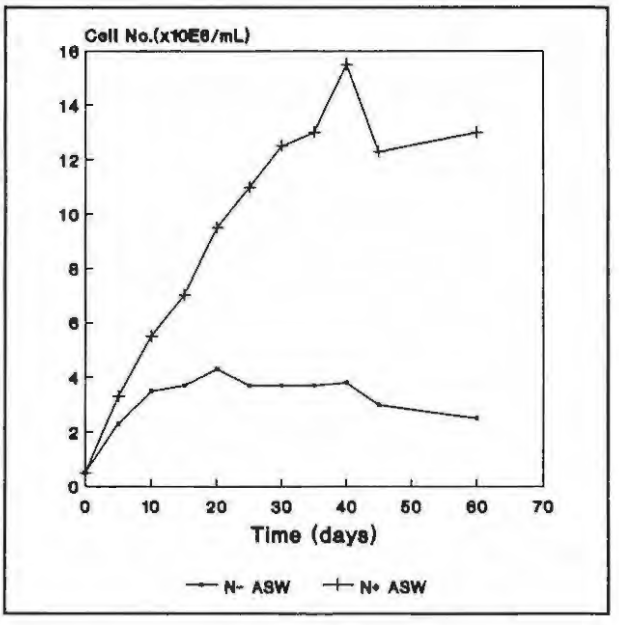


Figure 7.9 Growth of *D.salina* cells from a Hide Soak Liquor derived culture. Cells were washed, transferred to nitrogen-sufficient and nitrogen-deficient ASW medium and incubated for 60 days.

When *D.salina* cells, grown in Hide Soak Liquor, were actively separated and transferred to a nitrogen-deficient ASW stress medium, an increase in β-carotene was measured within the first few days. In this case, where β-carotene was measured as a function of culture volume, productivity was found to be about 30% higher than in the ASW-grown nitrogen-free controls at day 15 (figure 7.10). Figure 7.11 records the measurement of β-carotene production per cell for the same experiment. The initial rate of production in effluent-grown cells is

0.07pg.cell^{-1}.hour^{-1} compared to 0.21pg.cell^{-1}.hour^{1} for ASW-grown controls over the first three days. Rates equalize at about day seven at 0.12pg.cell^{-1}.hour^{-1}. This finding seems to provide further substantiation for the carry-over of stored intracellular nitrogen by cells grown in effluent.

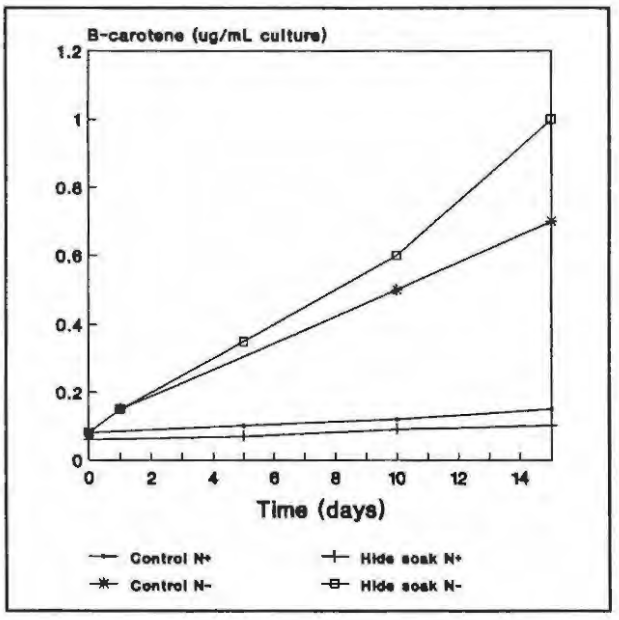


Figure 7.10 β-carotene production in Hide Soak Liquor and ASW-grown *D.salina*. Cells were washed and transferred to nitrogen-deficient ASW medium, and yield measured as a function of total culture volume.

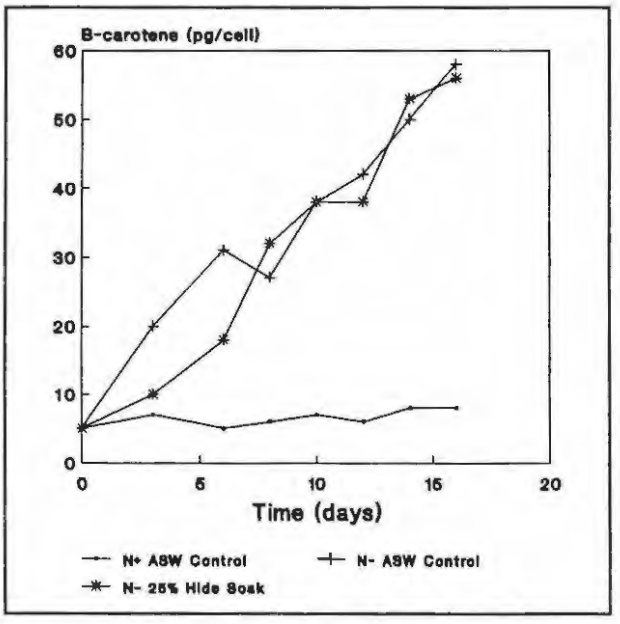


Figure 7.11 β-carotene production in Hide Soak Liquor and ASW-grown *D.salina*. Cells were washed and transferred to nitrogen-deficient ASW medium, and yield measured as pg.cell^{-1}.

7.5. DISCUSSION.

The results documented above seem to confirm, for ASW-grown pure cultures of *D.salina*, the previously reported findings in effluent-grown cells. Under conditions of low light, removal of nutritional nitrogen from the alga's medium would be essential to the satisfactory induction of β-carotene synthesis and its accumulation to appropriate levels (figures 3.5,3.6,3.10). It is also apparent that nitrogen-stress, if it can be practically applied in a metaholite accumulation stage separate from cell growth, would offer substantial advantages in terms of β-carotene yield compared to the use of only a salinity stress on its own (figure 7.4). Ben-Amotz and Avron (1983) previously observed that those stresses which caused a growth limitation produced similar effects in terms of β-carotene production. The results recorded here, however, rather tend to indicate a substantial difference in the β-carotene production effects caused by the various growth limiting stresses. A nitrogen-stress performed better than salt alone, and a combination of nitrogen and salinity stresses exert a synergistic effect on *D.salina*, producing an enhancement of β-carotene production larger than for each stress on its own.

The practicality of applying a passive cumulative stress to an HROP-grown culture of *Dunaliella* in an evaporation ponding cascade (an attempted simulation is reported in figure 7.8) is uncertain. While flask tests give very little indication of problems such as predation and competition pertaining in a large-scale operation, it is noteworthy that an Australian algal biotechnology company, Betatene Ltd., employs the extensive cultivation and harvesting of blooms of high β-carotene-containing *D.salina* directly from large salt lakes in South Australia (Schlipalius,1990).

In the simulation of the Active Multistage, the separation of effluent-grown cultures from the high-nitrogen growth media and transfer to nitrogen-deficient stress media produced satisfactory results in terms of total β-carotene production (figures 7.10, 7.11). As could be predicted from the earlier studies, the possible carry over of stored intracellular nitrogen by the effluent-grown cells produces additional cell growth and hence an overall superior final β-carotene yield.

7.5.1. A Nitrogen-stress Multistage Option.

While the β-carotene enhancement effects of a number of individual stress factors have been evaluated in the single stage "averaged" system and the use of salinity in a possible multistage approach has been discussed (Moulton *et al.*,1987a;Chen&Chi,1981), it appears that the manipulation of overall productivity in terms of enhanced cell number and β-carotene levels in a two stage process, using the regulation of nitrogen level (or any other nutritional stress rather than salinity alone), has not been previously considered. The potential yield advantages of nitrogen over salinity stress have been outlined above. While a theoretically derived yield enhancement that could be anticipated from such a system would be largely conjectural, this need not be entirely so. A first approximation based on a rough estimate of advantage, is possible using values derived from the literature and from the studies reported above. This comparison has been made in table 7.1.

Table 7.1 A comparison of the rates of β-carotene production induced by various stress factors, as derived from literature reports and in this study. All the data reflect synergistic stresses applied singly at 3M NaCl and at low light illumination which was generally about 100 $\mu mol.m^{-2}.sec^{-1}$. The Lers (1990) result for sulphate was recorded at $27\mu mol.m^{-2}.sec^{-1}$. The other exceptions were the salinity/high light stress reports which were measured at $2530\mu mol.m^{-2}.sec^{-1}$ (Ben-Amotz,1986) and $1650\mu moles.m^{-2}.sec^{-1}$ (Lers *et al.*,1990). The figures from Borowitzka *et al.*,(1990) were published as μg carotenoid.mg^{-1} cell protein and stresses from 1.7M to 3.4M NaCl. Normalization of these figures was based on a proportional adjustment of the cellular β-carotene level at 1.5M NaCl recorded in Figure 7.4 above. Rate as $pg.cell^{-1}.hour^{-1}$ was derived by dividing cellular yield by time. (n) indicates that the published result was normalised to 5 days, otherwise results were derived directly from published tables and graphs.

Stress	Cell	Yield-5 Days		Final Yield		Ref.
Type	$pg.cell^{-1}$	%dry wt.	rate (hr^{-1})	pg	days	
High Light	32	6.4	0.27	32	5	BA,1986
High Light	28	5.6	0.23	80	9	Lers,1990
Phosphate(n)	29	5.8	0.24	35	6	BA,1987
Nitrogen	28	5.6	0.23	40	10	Fig. 5.11
Nitrogen(n)	25	5	0.21	30	6	BA,1987
Sulphate(n)	20	4	0.17	16	4	Lers,1990
Sulphate(n)	17.5	3.5	0.15	21	6	BA,1987
3M NaCl	13.6	2.7	0.11	13.6	5	BA,1986
3.4M NaCl	13.5	2.7	0.11	16	7	BA,1990

Experiments chosen for the comparison were all performed at 3M NaCl which would approximate the underlying stress that would have to be applied in an open system to deal with the predation and competition requirements already identified. This implies that in all optimisation studies the stresses evaluated should be synergistic with high salt.

The comparative values for salt stress tabulated above are in general agreement with those recorded in Figure 7.4, including the Borowitzka *et al.* (1990) figures, the normalization of which is potentially the least accurate. The comparison shows that an elevation of salt levels provides the least effective stress method for the manipulation of cellular β-carotene. This indicates that the original consideration of the two stage process (Moulton *et al.*,1987a) designed around imposing a salt stress seems to have been based on a worst case evaluation. Discounting the predation control problem, and with the singular exception of the sulphate stress, the salt stress shows about half the potential of the other options for enhancing β-carotene accumulation at the 5 day period.

High-light stress, as previously reported, produces the most significant effect. The problem in practice is the inverse relationship that exists between cell number and light penetration into the ponds. The shading effects of dense cultures can be a problem even in areas of exceptionally high insolation (Borowitzka&Borowitzka,1988c;Richmond,1986d). Furthermore, high cell numbers have been identified as an important variable in reducing harvesting costs. Against this background, the ability to apply stress induction under conditions of low light appears to offer substantial advantages. The cellular yield of β-carotene with nitrogen and phosphate stresses, under low light conditions, was essentially comparable to the use of high light on its own. In addition, the five-day point considered for nitrogen-stress does not represent a ceiling and β-carotene productivities of between 8% and 10% cell dry wt. were recorded in figure 7.11 with 10-15 days of stress. Ben-Amotz (1986) shows similarly high longer-term accumulation values for nitrogen-deficient stress. These results were not included in table 7.1 since it appears that prestressed cells were used in his experiments which would have affected the initial rates. The additional potential of introducing a simultaneous phosphate stress in an effluent-based production system is also available, given the exceptionally low levels of this nutrient in tannery effluents (see table 4.1). The stress medium could be formulated quite simply to impose multiple nutritional stresses to act synergistically.

The above data also appears to allow a rough estimate of advantage for the nitrogen-stress multistage. Two options for the operation of the stress stage can be compared;

1. A short retention time stress unit (about 5 days) where lower cellular β-carotene values are accepted in favour of reduced retention times, which offers advantages of either increased throughput or reducing the size of the capital works requirement.

   By accepting β-carotene levels of 3%-5%, equivalent to those reported for the "averaged" open system (Ben-Amotz&Avron,1990), full advantage can be taken of the enhanced cell production that can be achieved under conditions of high nitrogen and moderate illumination in the initial growth stage of a two stage process (see figure 7.1). If the advantages of growth in tannery effluent or organic medium supplementation are taken into account (see figure 3.1) it appears that at least a doubling of cell number in the first stage is quite feasible.

2. A longer retention time stress unit (10 days plus) could allow douhling, to about 10% of cell dry wt, of the final cellular β-carotene yield currently achieved in the single stage systems.

   Considering simultaneous cell growth and β-carotene enhancement effects together, this rather provisional benefit analysis demonstrates a theoretical potential of a four-fold yield enhancement that could be derived from a separation of growth and β-carotene accumulation into two separately optimized unit operations. Assuming a worst case cost scenario, with a

requirement for two harvests and a doubling of all other costs, the additional yield would still provide a two-fold improvement in the yield:cost ratio.

While the provisional nature of the above estimates should again be stressed, the potential suggested appears to warrant the further evaluation of a nitrogen stress-based multistage approach to β-carotene production from mass cultures of *D.salina*, whether grown in effluent or in defined culture media. In any event, it makes a virtue of necessity, in the case of an effluent-based process, where β-carotene production could not be practically considered in the absence of the separation of growth and stress stages.

CONCLUDING REMARKS.

The advantages identified for the nitrogen-stress multistage appear to be of equal application to both defined medium and effluent based *D.salina* culture systems. Development of micro-algal harvesting technology will be needed, given the crucial requirement for the separation of cells from their growth medium. In the case of effluent-grown cells, where intracellular accumulation of nitrogen stores will defer the β-carotene induction process for an unspecifiable time, a more precise monitoring of the initiation of the stress response will be required as apposed to merely waiting for the accumulation of the product. The following chapters will deal with the requirements of stress initiation.

CHAPTER 8

THE HORMONAL MEDIATION OF STRESS IN DUNALIELLA

Summary.

Evidence is presented which strongly suggests a hitherto-unreported role for the plant hormone, abscisic acid, in the mediation of the response of *D.salina* to a number of stress conditions. Photosynthetic data indicate the presence of functional sensitivity in *Dunaliella* controlled hy a signal response mechanism common to the different stress forms examined. The stress response appears to be regulated through four clearly defined steps, namely the signal, ABA accumulation, and early and late β-carotene accumulation. It is noted that monitoring of the stress induction event offers the prospect of a more precise control of the biotechnology of metabolite accumulation in *D.salina*.

8.1. INTRODUCTION.

Stress is the result of any constraining force or influence which limits the normal growth and development of an organism, and in nature is ever-changing, erratic or unevenly cyclical (Seeley,1990). This in turn is reflected in physiological responses, of which the final effect are characteristic of the particular stress imposed. The significance of stress factors on the accumulation of the metabolites β-carotene and glycerol by *D.salina*, has already been noted. Successful manipulation of these stress factors, to enhance the production of metabolites, is likely to depend on a clearer understanding of the regulation of the stress event at the physiological and molecular level, especially where the synergistic effects of multiple stresses can impose additional complexity on biotechnological management. In this regard signalling mechanisms that initiate physiological stress responses could be of particular significance in the design and operation of a production process, the optimization of which depends on the separation of cell growth and metabolite induction into different unit operations. The need for a sensitive indicator of product induction is especially apparent where the accumulation of nitrogen by *D.salina*, grown in proteinaceous tannery effluent, serves to suppress the intensity of the β-carotene production response. It could be anticipated in this case that the imposition of stress and the initiation of product accumulation could be separated by days, or even weeks in a large scale operation. Specific intervention in the production process would require knowledge of the signal marking the actual commencement of the response.

8.1.1. The Plant Hormone - Abscisic Acid.

Evidence that the physiological responses of *Dunaliella* to environmental stresses, such as glycerol production with salinity shock (Ben-Amotz&Avron,1973), and β-carotene accumulation with high-intensity irradiation (Ben-

Amotz&Avron,1983,1989a) function as mechanisms which serve to protect the organism from the particular stress raises the possibility that these responses may be mediated by hormonal activity.

The role of the plant hormone abscisic acid (ABA) in signalling and mediating stress responses in higher plants has been the subject of intensive study over the last two decades and has been reviewed by Walton (1980), Sembdner *et al.* (1980), Addicott (1983) and Zeevaart and Creelman (1988). The action of ABA was originally considered to be that of a growth inhibitor, controlling leaf abscision and dormancy in seeds and buds. It is now known to have multiple roles in higher plants including regulating root growth, respiration, fruit ripening and senescence, where it is also associated with the accumulation of carotenoid pigments (Owen&Napier,1988). In addition, the apparent ubiquitous involvement of ABA in the mediation of stress responses in higher plants, particularly to salinity and dehydration, has led to its being commonly known as the "Stress Hormone" (Zeevaart&Creelman,1988).

8.1.2. A Role for ABA in *Dunaliella*.

The presence of ABA in algae has been suspected for many years (Jennings,1969;Pryce,1972;Niemann& Dorfling,1980), and the effects of exogenous applications of ABA to the algae include growth inhibition of *Ecklonia* gametophytes (Jennings,1969) and effects on ATP levels in *Scenedesmus* (Tilherg,1970). The unequivocal demonstration of the presence of the hormone in green algae was reported by Kingsham and Moore (1982), Tietz and Kasprik (1986) and Sabbatini *et al.* (1987). Since then surveys have shown its universal distribution within the Phycophyta including the first reports of ABA in *Dunaliella* by Tietz *et al.* (1989) and Hirsch *et al.* (1989).

ABA has been shown to accelerate the adaptation of tobacco cells to salinity stress (La Rosa *et al.*,1987). Creelman (1989) has reviewed inhibitor and mutant studies which indicate that the inability to accumulate ABA is associated with carotenoid deficiency in plant cells. An equivalent physiological role for ABA as a stress hormone, active in the algae, would require the demonstration of more than its mere occurrence, in order to confirm a functional hormonal role for the substance in regulating algal metabolism.

Requirements for such a demonstration have been noted by Tietz *et al.* (1989) and Hirsch *et al.* (1989):

1. A specific mechanism should exist whereby the cell is able to regulate endogenous levels of ABA by both the rates of synthesis and degradation of the compound.
2. A change in endogenous ABA levels should be correlated with one or multiple physiological responses which enable the cell to cope with a particular stress condition.

Tietz *et al.* (1989) reported both the intracellular accumulation and release of ABA to the medium in response to salinity stress, and speculated on a role for ABA in regulating stress in the algae in a similar way to that already reported in higher plants. Hirsch *et al.* (1989) have further demonstrated the synthesis of [^{14}C]-ABA

from externally applied [^{14}C]-mevalonic acid and its degradation to phaseic acid (PA) and dihydrophaseic acid (DHAP), which points to a common origin for ABA and carotenoid in *Dunaliella*. Mevalonate is the precursor of the terpenoid pathway which leads to the production of carotenoids (Creelman,1989). This together with the inhibition of ABA production by norflurazon, an inhibitor of β-carotene in *Dunaliella* (Ben-Amotz *et al.*,1987), further points to a common origin for ABA and carotenoid in this organism. Recent evidence from studies using higher plant tissues favours the biosynthesis of ABA from all-*trans* violaxanthin, a process involving cleavage of *cis* neoxanthin to yield xanthoxin which is subsequently converted to ABA via ABA aldehyde (Parry *et al.*,1990). This proposed biosynthetic pathway for ABA in higher plants emphasizes the possible interrelationship that could exist between β-carotene accumulation and ABA production in *D.salina* cells exposed to stress.

The demonstration of the accumulation of ABA in response to salt stress by both groups of authors provides good circumstantial evidence for a hormonal role for this substance, but Hirsch *et al.* (1989) were unable to link the accumulation of ABA with any specific physiological effect. Hirsch *et al.* (1989) furthermore addressed the prospect that in the absence of a demonstrable role for ABA recently identified in the algae, its presence could be interpreted as the by-product of certain metabolic reactions which developed a functional phytohormone role only later in plant evolution. This raised the question whether the compound isolated from *D.salina* was, in fact, biologically active. Although it had been structurally confirmed by Gas Chromatography-Mass Spectrometry by Tietz *et al.* (1989), separation of the stereoisomers of ABA would be necessary to determine whether it was present in the active ABA (+)-S form. Until recently the algae had been thought not to contain ABA at all, but rather lunularic acid, a C15 compound with a structure similar to ABA (Owen&Napier,1988).

It seemed that an appropriate demonstration of biological activity in an algal extract should precede attempts to link ABA accumulation with a specific physiological response in the algae. The original demonstrations of the biological activity of ABA, as an indole acetic acid (IAA) antagonist, had been made in coleoptile bioassays (Millborrow,1967). The use of the *Triticum* coleoptile bioassay for ABA has been reported by Nitsch and Nitsch (1956), Hancock *et al.* (1964), Taylor and Burden (1972). Reeve and Crozier (1980) have reviewed the use of bioassays in the quantitative and qualitative analysis of plant hormones, and conclude that, despite distinct limitations compared to the potential of physico-chemical methodology, the demonstration of biological activity and the simplicity of the bioassay ensures its continued use as an analytical tool. The demonstration of biological activity in an algal extract applied to a higher plant system would add further weight to the assumption of a hormonal role for the substance identified as ABA by Tietz *et al.* (1989) in *Dunaliella*.

8.1.3. Sensitivity.

The previously established methodologies for demonstrating physiological responses for hormones in plant systems have been questioned. Trewavas (1981) drew attention to observations that hormonal concentration alone was not sufficient to account for the effects observed in higher plants, and that the response involved another factor which he called sensitivity. This concept implies the presence of receptor sites, the active number of which will dictate the intensity of the particular response. In this way changes in the responsiveness of plant cells and tissues would be more important than changes in the concentration of the hormone itself. An analogy has been drawn with the concept of parameter sensitivity used in control and system engineering.

The controversy that followed this proposal has been reviewed by Trewavas (1991) together with the numerous reports where sensitivity has been demonstrated to play a role in the regulation of higher plant development. The use of the term hormone has itself been questioned, given the demonstrable differences comparing plant to animal systems, where responses are concentration related. The term growth substance has been preferred, but this again implies structural differentiation effects in higher plant tissues which are not comparable in a unicellular system. While bearing these distinctions in mind, the objective here is not to enter the terminological debate, and for purposes of the subsequent discussion the term plant hormone has been retained.

Trewavas (1991) has argued that sensitivity should be seen within the holistic network of interrelated biochemical and physiological processes, and that relevant changes in hormone concentration should be related to small variations around endogenous levels. His systems view is contrasted with a more simplistic notion of control where the activity of certain "critical" molecules has been demonstrated, sometimes under extreme experimental conditions, with distortions resulting from the use of inhibitors, mutants and saturating levels of exogenously applied hormone. This has led, in the view of Trewavas, to a confusion of a part of the mechanism of developmental change as its control.

Ideally then, the demonstration of control exerted by any one constituent molecule should not only be equated with the size of a physiological response when it is added to a system at realistic levels, but an endogenous response should be demonstrated dependent on a precise set of environmental, developmental and experimental conditions. The experimental design that follows has attempted to take into account the requirements set out by Trewavas (1991) for the unambiguous demonstration of hormonal sensitivity. These include the avoidance of artifacts by manipulation at the endogenous level, constraints on experimental manipulation with non-invasive measurement using whole cell systems, and the contribution to control, measured as a function of the interacting components of the physiological response.

8.2. RESEARCH OBJECTIVES.

1. To determine the presence of appropriate biological activity in an extract of *D.salina* cells as applied in a wheat coleoptile bioassay;
2. To asses the metabolism of ABA in *D.salina* with reference to the synthesis and degradation of the compound;
3. To evaluate an interrelationship between changes in endogenous levels of ABA and β-carotene accumulation in cells of *D.salina* exposed to salinity stress;
4. To demonstrate the presence of an actual stress effect on the basic physiology of the alga by hypersaline exposure, as reflected by changes in CO_2 fixation and productivity.
5. To attempt to relate the changes in endogenous levels of ABA to physiological responses reflected by the photosynthetic apparatus.

8.3. MATERIALS AND METHODS.

8.3.1. Bioassay.

Cells of *D.salina* were grown in ASW medium as previously described and salt shocked by raising salinity to 3.5M NaCl 24 hours before harvesting. Cells were separated from the growth medium by centrifugation and washed twice in fresh ASW medium. The methanolic extract was prepared by rupturing the cells in distilled water, followed by the addition of 80% methanol and permitting extraction at 4°C overnight. The aqueous extract was prepared by cell rupture in distilled water followed by similar extraction. Extracts were clarified by centrifugation at 10 000 g for 15 minutes at 4°C.

Four-day *Triticum* coleoptiles were cut to 1cm lengths at least 1cm below the tip, soaked in distilled water and distributed 10 to a petri dish. Hormone standards abscisic acid (ABA), benzyladenine (BA), gibberellic acid (GA3) and indole acetic acid (IAA) (Sigma) were diluted in distilled water over the range 10^{-4} to 10^{-7}M and cell extracts in the range undiluted, 1/2, 1/4,1/8 in distilled water were added to petri dishes at a final volume of 10mL. All procedures were carried out in the absence of white light. Tests were incubated in the dark at 21°C for 24 hours when the coleoptile lengths were measured.

8.3.2. Application of Radiochemicals and Incubation Procedures

Cells of *D.salina* were harvested by centrifugation at 20°C in a Sorvall RC-5 refrigerated centrifuge at 3000g for 10 minutes. The algal pellet was washed in 1.5M KCl to reduce "clumping" and recentrifuged at 3000g for 10 minutes. The washed pellet was resuspended in fresh growth medium and, unless otherwise stated, 6mL (equivalent to $42x10^6$ cells) were incubated in 25mL Erlenmeyer flasks containing either R-[2-^{14}C]Mevalonolactone (MVAL) (specific activity 2.03GBq.mmol^{-1}). NaH[^{14}C]O_3 (specific activity 1.91 GBq.mmol^{-1}), or (R,S,)-[2-^{14}C] ABA (specific activity 1.30 GBq.mmol^{-1}), which were all obtained from Amersham. Incubations were carried out in a shaking reaction incubator at 25°C under continuous low-light illumination (42μmol.m^{-2}.sec^{-1}). After 20 hours the uptake of radioactive substrates was stopped by

centrifugation and repeated washing (three times) of cells in equal volumes of 1.5M KCl. Pellets were resuspended in fresh growth medium containing 3M NaCl, and incubated as above for various lengths of time as specified in "Results". Incubations were terminated by centrifugation and washing in fresh culture medium of identical NaCl molarity. Chlorophyll content was determined (Arnon,1949) and cells, incubation media, and washings were extracted and analyzed for ABA and its acidic products as described below.

8.3.3. Incubations with Inhibitors.

Cycloheximide ($0.2\mu g.mL^{-1}$) and chloramphenicol ($50\mu g.mL^{-1}$) were supplied to low-light illuminated cells 4 hours before exposure to salinity stress, and actinomycin D ($50\mu g.mL^{-1}$) was added 12 hours before exposure (in each case final concentration is indicated). Incubation flasks containing chloramphenicol or actinomycin D and their respective controls were covered with yellow cellophane as described by Lers *et al.* (1990).

8.3.4. Extraction, Purification and Analysis of Radioactive ABA and PA.

Algal material, harvested by centrifugation, was usually extracted by sonication in ice-cold methanol/ethyl acetate (50:50v/v) containing butylated hydroxytoluene ($20mg.L^{-1}$). Extracts were filtered through Whatman No.1 filter paper, and the residue was washed with excess solvent. The filtrate was reduced to dryness *in vacuo* at 35°C and resuspended in 0.5M potassium phosphate buffer (pH 8.5). The aqueous phase was partitioned three times against equal volumes of diethyl ether to remove neutral and basic impurities. Aqueous extracts were then acidified to pH 2.5 and partitioned three times against equal volumes of ethyl acetate to extract the acids. Water was removed by freezing and filtration, and the ethyl acetate-soluble acids were applied to a pre-rinsed Sep-Pak C_{18} cartridge (Waters). Incubation media containing the respective washings were acidified to pH 2.5 and likewise partitioned three times against equal volumes of ethyl acetate. These were also applied to a pre-rinsed Sep-Pak C_{18} cartridge. ABA and PA were eluted from the cartridges using a profile similar to that described by Pierce and Raschke (1981). Purified samples were chromatographed on thin layers of silica gel GF/254 (Merck) developed twice to 15cm in toluene/ethyl acetate/acetic acid (25:15:2v/v/v). Plates were air dried, divided into 30 equal strips, and eluted with absolute methanol, and the levels of radioactivity were determined in a Beckman LS 5801 scintillation counter, programmed for automatic quench correction, following addition of 10mL of cocktail (2,5-diphenyl oxazole pyrophosphate in toluene $5g.L^{-1}$). Radioactive ABA and PA were identified according to the criteria of Millborrow (1974) and Zeevart and Millborrow (1976) using biosynthetically prepared PA (Cowan&Railton,1987) and authentic ABA (Sigma) as markers. Putative ABA and PA, well resolved by TLC in toluene/ethyl acetate/acetic acid (25:15:2v/v/v), were eluted with water saturated ethyl acetate and methylated with ethereal diazomethane and re-chromatographed by TLC in hexane/ethyl acetate (1:1v/v). After re-elution with ether, methylated ABA was reduced to an equal mixture of the 1',4'-*trans* and 1,4-*cis* diols using $NaBH_4$ in aqueous methanol at 0°C, which was then separated by TLC in benzene/ethyl acetate/acetic acid (15:3:1v/v/v). Likewise, methylated PA was reduced to an equal mixture of dihydrophaseic acid and its 4'-epimer, following treatment with $NaBH_4$, which was then resolved by TLC in hexane/ethyl acetate (1:1v/v), and the plates were developed three times to 15 cm.

8.3.5. Determination of ABA and β-carotene.

To enable appropriate comparisons to be made it was important to measure levels of ABA and β-carotene in the same extract. Once harvested, cells were resuspended in 50mL of fresh growth medium, containing 3M NaCl, to give a culture density of $13x10^4$ cells.mL^{-1}. Cells were incubated as described above. Cell cultures of the same density growing in 1.5M NaCl medium were included as controls. At each assay interval, aliquots were removed for the determination of chlorophyll (Arnon,1949) and dry weight. Cells were extracted for ABA and β-carotene in ice-cold acetone (-20°C) containing 2,6-Di-*tert* butyl-p-cresol (butylated hydroxytoluene, 20mg.L^{-1}) added as an antioxidant, by sonication and stored at -20°C under nitrogen in the dark until analyzed.

For analysis of β-carotene, acetone extracts were reduced to dryness, re-dissolved in 70% methanol, and applied to Sep-Pak C_{18} cartridges. Oxygenated carotenoid were eluted with 5mL 90% methanol, chlorophyll with 5mL 100% methanol, and carotene with 5mL 100% acetone as described by Eskins and Dutton (1979). The concentration of β-carotene in the acetone elutes was determined according to the method of Ben-Amotz and Avron (1983).

The levels of endogenous ABA were determined in similar acetone extracts containing a known amount of high specific activity [^{3}H] ABA (added to correct for recovery) and ABA-methyl ester (Sigma), as an internal standard. Acetone was removed *in vacuo* at 35°C and the aqueous residue purified using Sep-Pak C_{18} cartridges as described above. The incubation media and washings were similarly purified following acidification. Aliquots of these elutes were analyzed by reverse-phase HPLC using a 0 to 100% methanol gradient containing 0.5% acetic acid throughout and quantified by peak integration and calibration with reference compounds. The liquid chromatography apparatus consisted of a high-pressure ternary pump (Spectra-Physics SP 8800), a Rheodyne 7125 injector (20μL loop), and a 5μ ODS Spherisorb column (4.6x100mm i.d.). Flow was 1mL.$minute^{-1}$, and peaks were monitored at 254nm with a linear UV06 fixed wavelength detector coupled to a Spectra-Physics SP 4290 integrator. The fractions corresponding to authentic ABA and PA were pooled, reduced to dryness, and methylated with excess ethereal diazomethane. Unequivocal identification of these products as ABA and PA was established by combined capillary GC-MS.

GC-MS was performed on a Hewlett-Packard 5890 instrument, using a fused-silica capillary column (12mx0.32mm i.d.) of HP-1 programmed from 120°C at 5°C.$minute^{-1}$ with helium as the carrier gas (1.5 mL.$minute^{-1}$), coupled to an HP 5988A MS system. Electron impact mass spectra were recorded at 70eV and a source temperature of 250°C. Identification of the compounds as methylated ABA and methylated PA was established using an Hewlett-Packard data processing station.

8.3.6. Stress Induction of Chlorophyll Fluorescence.

The measurement of chlorophyll fluorescence was used to monitor the effects of stress on the photosynthetic apparatus. The procedure used here was the same as that described in 5.3.4.

Various stress conditions were applied as described below and the traces recorded are characteristic of at least three repetitions of each experiment.

8.3.7. Photosynthetic Carbon Fixation.

The rate of carbon fixation was measured to monitor the effects of stress on photosynthesis. A log phase culture of *D.salina* grown under a medium intensity light regime (147μmol.m^{-2}.sec^{-1}) was centrifuged at 3000g for 10 minutes, washed twice in 1.5M NaCl, resuspended in fresh nitrogen-sufficient or deficient ASW medium at 1.5M and 3M NaCl as required, at a cell density of 1x10^{4}.mL^{-1} and split into 4x50mL aliquots in 100mL flasks. 37kBq.mL^{-1} NaH[^{14}CO]$_3$ (Amersham) (specific activity of 1.91 GBq.mmol) containing 1.64μg carbon per 37kBq was added to each flask and the first 5mL sample withdrawn after 5 minutes. Flasks were then exposed to continuous illumination with low light (100μmol.m^{-2}.sec^{-1}) supplied by cool white fluorescence tubes, and to high light (1500μmoles.m^{-2}.sec^{-1}) supplied by 4x500 watt tungsten lamps. Temperature was regulated at 30°C. Samples were drawn after 5 minutes and again at 6 and 24 hours and centrifuged at 3000g for 10 minutes. Cells were then washed twice in fresh ASW medium, resuspended in 0.5mL distilled water and transferred to aqueous scintillant (Packard.) Counts (dpm) were recorded using a Beckman LS 315 OT scintillation counter.

Total inorganic carbon available as CO_2 was determined by acidification using the method of Lewis and Smith (1983). Measurement was made by injection of 100μL of generated headspace gas onto a Poroplot Q 25M capillary GC column (Supelco) in a Chrompak 9000 gas chromatograph. The amount of carbon fixed was determined according to Gocke and Hoppe (1978). Results reflect the mean of three replicates.

8.4. RESULTS.

8.4.1. Bioassay.

The bioassay results are reported in table 8.1 with both aqueous and methanolic extracts of salt stressed *D.salina* showing an ABA-like inhibition of *Triticum* coleoptile growth of 20% and 18% respectively. The concentration of the inhibitory substance present in the algal extract (355pg.mg dry wt.-1), was calculated against the bioassay standards. Given the limitations generally accepted for the method, it appears reasonable to conclude that a biologically active plant growth inhibitor(s), consistent with the occurrence of ABA, is present in the extracts.

Table 8.1 *Triticum* coleoptile bioassay recorded as percentage change in length at 24 hours. Dilutions of methanolic and aqueous extracts were assayed against abscisic acid (ABA), benzyladenine (BA), gibberellic acid (GA3) and indole acetic acid (IAA) standards.

CONC.	ABA	BA	GA3	IAA	CONC	Me.ext	Aq.ext
10^{-4}M	10.7	18.3	13.8	25.4	undil	9	5.7
10^{-5}M	13	20	18.3	25.4	1/2	14.5	13
10^{-6}M	16.6	22.4	23.7	25.4	1/4	14.5	15
10^{-7}M	18.3	23.6	24	23.7	1/8	18.7	20.6

8.4.2. ABA Metabolism in Salt Stressed Cells.

Analysis of the Sep-Pak C_{18}-purified acids from both stressed and non-stressed cells supplied with R-[2-^{14}C]MVAL, and the respective incubation media, resulted in the distribution of radioactivity show in Figure 8.1. The data from this experiment allow several observations to be made:

1. Cells of *D.salina* are able to transform labelled MVAL into ABA and PA, suggesting that both the ABA anabolic and catabolic pathways are operational in this organism.
2. Both processes are enhanced by hypersaline conditions.
3. Cells do not appear to accumulate appreciable amounts of either ABA or PA in response to salt stress.
4. Any ABA and PA produced is preferentially partitioned into the culture medium, and this partitioning effect increases in response to salt stress.

Thus, the overall effect of hypersalinity stress on ABA metabolism in cells of *D.salina* would appear to be enhancement, manifested by accumulation of ABA and PA in the culture medium. To investigate this aspect in more detail the rate of appearance of ABA in the culture medium was determined. Furthermore, a kinetic study was carried out to determine the distribution of labelled ABA between cells and the incubation medium.

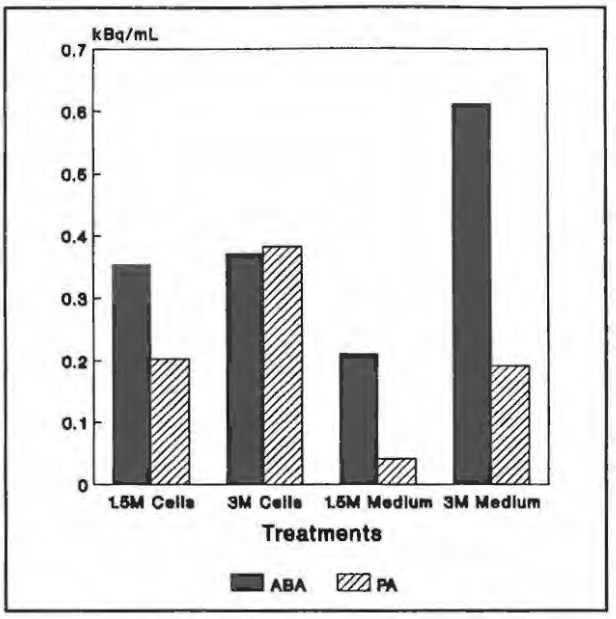


Figure 8.1. The synthesis and metabolism of ABA by cells of D.salina exposed to different salinities. Aliquots of cells (6mL, equivalent to 0.145mg.chl*a*.mL^{-1}) at a culture density of 7x10^{6} cells.mL^{-1} were supplied R-[2-^{14}C]MVAL (366.6 kBq) and incubated for 20 hours at 25°C before exposure to hypersaline conditions. Following pre-incubation in 1.5M NaCl medium, cells were washed by centrifugation and resuspended in fresh medium containing either 1.5M NaCl (for control cells) or 3M NaCl (stressed cells) and incubated for an additional 4 hours at 25°C. Cells were separated from the medium by centrifugation, and [^{14}C]ABA and [^{14}C]PA were extracted and analyzed. Data are expressed as the mean of three replicates.

The results presented in Figure 8.2 show that the rate of appearance of labelled ABA in the incubation medium increased dramatically within 30 minutes of exposure to salinity stress. Thereafter, the rate of appearance of ABA declined to levels similar to those observed in the control. Detailed kinetic studies (Figure 8.3) revealed that with time the amount of labelled ABA in the incubation medium increased and that the rate of appearance was similar to that observed in Figure 8.2. In contrast, levels of radioactive ABA associated with the cells did not change significantly during the course of incubation. However, a slight increase was observed immediately after the imposition of hypersaline conditions. This increase appeared to suggest an alteration in the levels and/or activities of the ABA-biosynthesizing enzymes induced by hypersaline conditions. To elucidate this aspect further, transcriptional (actinomycin D) and translational (chloramphenicol and cycloheximide) inhibitors were added at the appropriate time and at concentrations known to inhibit the induction of β-carotene accumulation (Lers *et al.*, 1990).

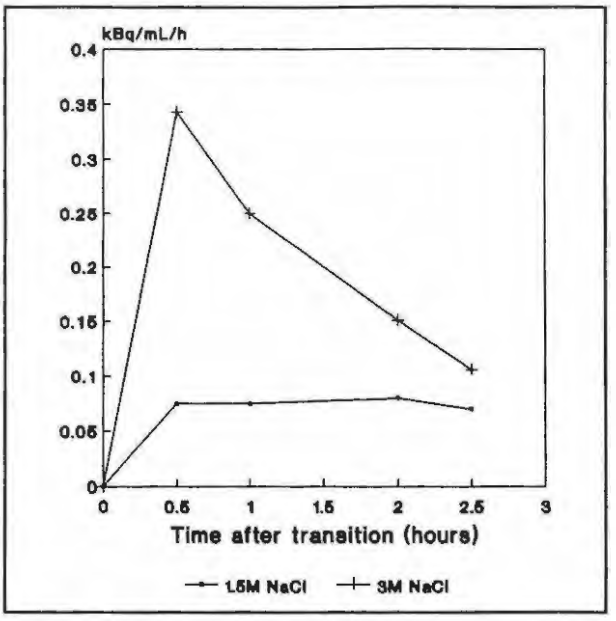
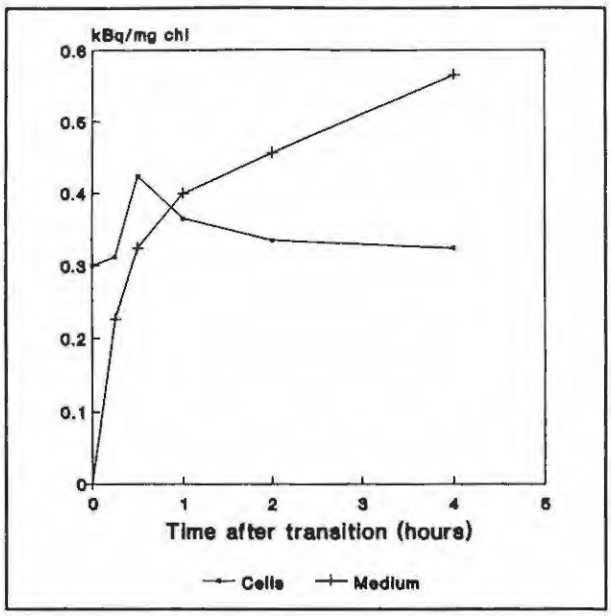
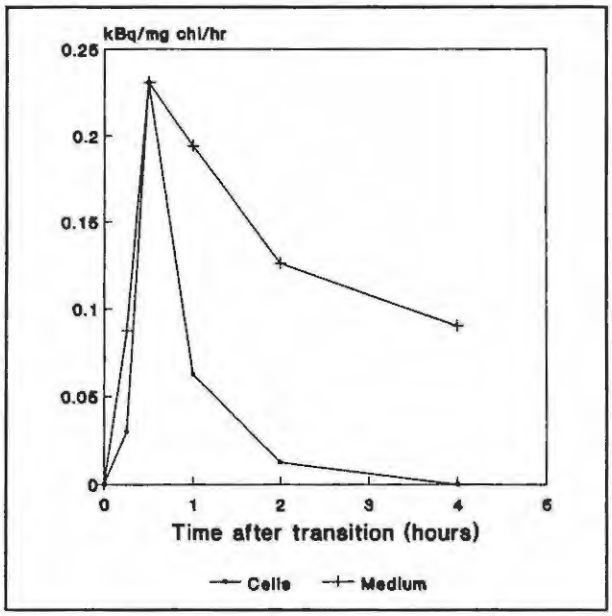


Figure 8.2. The rate of appearance of ABA in the culture medium from cells of *D.salina* following exposure to salinity stress. Aliquots of cells (4mL, equivalent to 0.25mg.chl*a*.mL^{-1}) at a culture density of 9x10^{6} cells.mL^{-1} were supplied with R-[2-^{14}C]MVAL (366.6 kBq) for 20 hours and then transferred to fresh medium containing either 1.5 or 3M NaCl for 3 hours. Cells were removed by centrifugation, and the amount of [^{14}C]ABA present in the culture medium was determined. Rates were calculated by subtracting the initial [^{14}C]ABA amount from the amount of [^{14}C]ABA at the end time point of each interval. This value was expressed as a function of time in hours and plotted against the time point of each assay interval. Conditions of incubation and analysis were as described in figure 1. Data are representative of two independent experiments.

Hypersalinity induction of labelled ABA production was inhibited by actinomycin D, cycloheximide and chloramphenicol (table 8.2). These data therefore indicate that both transcription and *de novo* protein synthesis are required for the increased production of ABA induced by hypersalinity. Furthermore, the results in table 8.2 show that incorporation of label into ABA was similar for cells supplied with either NaH[^{14}C]O_3 or R-[2-^{14}C]MVAL. This coupled with the inhibition of ABA biosynthesis by chloramphenicol strongly suggests that plastid-localized enzymes are involved in the induction of ABA production by cells of *D.salina* exposed to hypersaline conditions.



(A)



(B)

Figure 8.3. Time course of ABA accumulation and rate of production in cells and culture medium following exposure of *D.salina* to salinity stress. Application of R-[2-^{14}C]MVAL and incubation conditions were as described in figure 8.1 (A).Distribution of [^{14}C]ABA between cells and culture medium during the first 4 hours after exposure. (B).Rate of [^{14}C]ABA production during the 4 hour stress period. Appearance rates were calculated as described in figure 8.2. Data are representative of two independent experiments.

Table 8.2 Effect of inhibitors of translation and transcription on the incorporation of label from either [2-^{14}C]MVAL or NaH[^{14}C]O_3 into ABA by cells of *D.salina* exposed to salinity stress. Aliquots of the algal culture (6mL, equivalent to 42x10^6 cells) were supplied with either R-[2-^{14}C]MVAL or NaH[^{14}C]O_3 (both 366.6 kBq) and incubated at 25°C under continuous low illumination (42μmol.m^{-2}.sec^{-1}). Inhibitors of transcription and translation were applied at the appropriate time as described in "Materials and Methods", after which cells were exposed to increased salinity for 4 hours (from 1.5 to 3M NaCl medium) and ABA was extracted and analyzed as described in figure 8.1.

Treatment	NaH[^{14}C]O_3	[2-^{14}C]MVAL
	Bq.mL^{-1}Chl*a*(%)*	
Control	386.2(0)	428.7(0)
Actinomycin D (50μg.mL^{-1})	164.4(57.4)	166.1(61.3)
Cycloheximide (0.2μg.mL^{-1})	185.9(62.2)	151.1(64.8)
Chloramphenicol (50μg.mL^{-1})	171.8(55.5)	194.3(54.7)

8.4.3. Kinetics of ABA and β-carotene Accumulation.

The results presented in figure 8.4 show that in response to stress, the β-carotene content of cells of *D.salina* declined within 6 hours but had returned to basal levels 24 hours after the imposition of stress. Thereafter, the concentration of β-carotene in non-stressed cultures remained constant, whereas β-carotene in stressed cells began to accumulate by day 3. The early decline in β-carotene content immediately following the imposition of stress might reflect a stress situation caused by centrifugation during transfer of cells. However, the decline in β-carotene content was less marked in control cells, suggesting that this response was, at least in part, due to increased salinity.

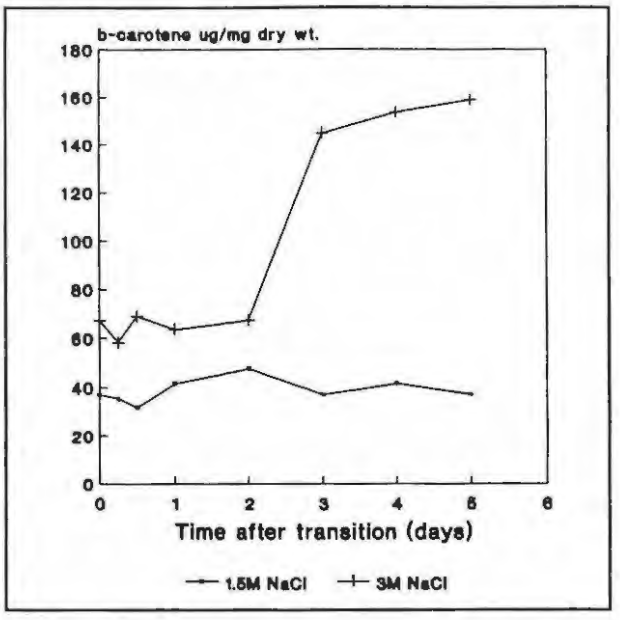


Figure 8.4 Kinetics of β-carotene accumulation by cultures of *D.salina* exposed to increased salinity. After harvest, 50mL aliquots of cells at a culture density of $13x10^6$ cells.mL^{-1} were incubated in either 1.5 or 3M NaCl medium at 25°C. β-carotene content was determined after extraction of cells in acetone. Data represent the mean of three replicates.

Oxygenation of carotenes yields xanthophylls such as violaxanthin and neoxanthin, the postulated precursors of ABA (Zeevaart&Creelman,1988). No change in bulk oxo-carotenoid content was detected in cultures exposed to either 1.5 or 3M NaCl (data not shown). However, a significant increase in levels of ABA was detected in cultures of *D.salina* exposed to 3M NaCl (figure 8.5). ABA levels reached a maximum 8 hours after exposure to hypersaline conditions and then declined to a basal level of 3 ng.mg^{-1} dry weight. A similar but less dramatic response was detected in control cultures at 1.5M NaCl, again suggesting a stress condition imposed by centrifugation and cell transfer. Nevertheless, the massive accumulation of ABA in cells exposed to hypersaline conditions strongly suggests that ABA production increased in response to salt stress. Moreover, the ABA content of the medium from cells incubated at 3M NaCl increased throughout the incubation period, whereas ABA levels in the medium from control cells did not change significantly (figure 8.6). These data indicate that under conditions of hypersalinity there is an immediate response in terms of increased ABA production and that the bulk of this ABA is partitioned into the culture medium.

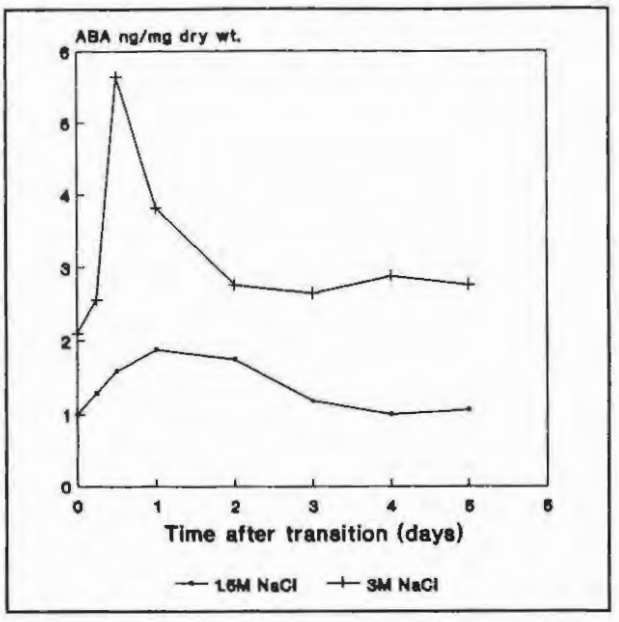


Figure 8.5 Kinetics of ABA accumulation by cells of *D.salina* exposed to increased salinity. ABA levels were monitored following transfer of cells to fresh incubation medium containing either 1.5 or 3M NaCl. Conditions of incubation are as described for figure 8.4. Data represent the mean of three replicates.

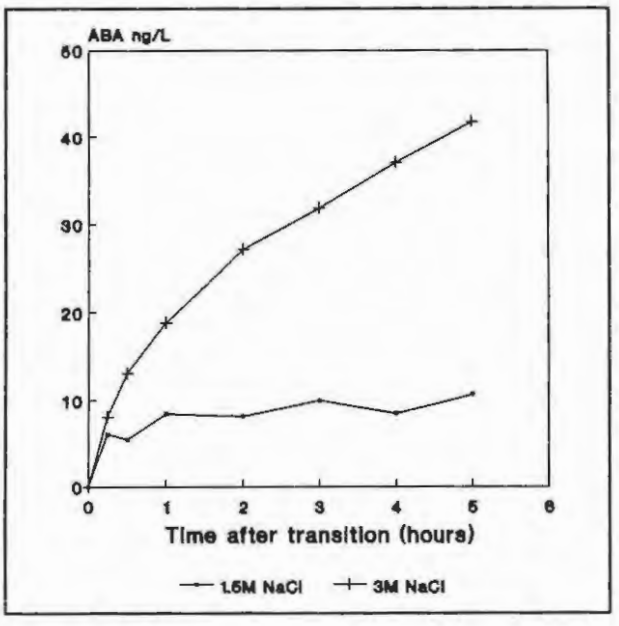


Figure 8.6 Course of ABA accumulation in the culture medium from cells of *D.salina* exposed to increased salinity as described for figure 8.5. Cells were transferred to fresh medium (see figure 8.4) containing either 1.5 or 3M NaCl, and levels of ABA were monitored during a 5 day period. Data represent the mean of three replicates.

In attempting to elucidate the interrelationship between β-carotene accumulation and ABA production, the rate of change in both β-carotene and ABA production was calculated. The result shown in figure 8.7 indicates two

stages of accelerated β-carotene accumulation and that the initial stage of increased β-carotene production coincides with the only stage of accelerated ABA production.

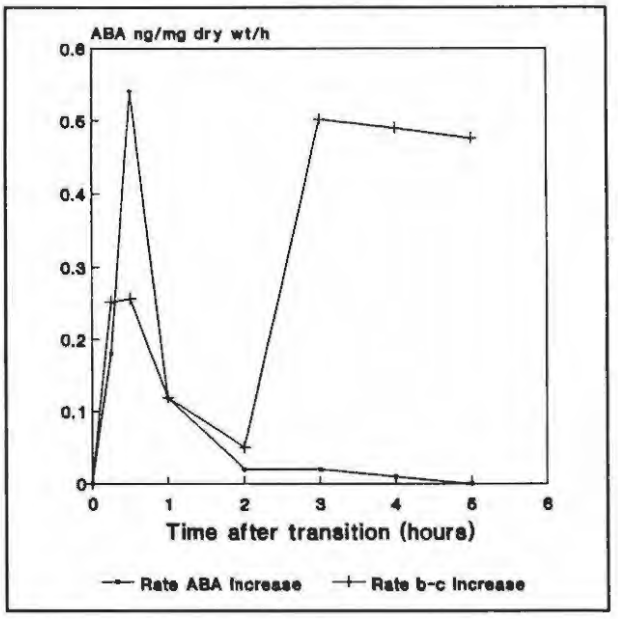


Figure 8.7 The rate of β-carotene accumulation by cells of *D.salina* exposed to hypersaline conditions.Rate of accumulation was calculated from each time point (figures 8.4 and 8.5) by subtracting the initial concentration from the final concentration divided by the time taken to reach the final concentration. These values were plotted against the end time point of each time interval. Data represent the mean of three replicates.

8.4.4. Photosynthetic Stress Response - Fluorescence Induction.

The effect of various stresses on the photosynthetic apparatus of *D.salina* was monitored by measuring changes in chlorophyll fluorescence and the carbon fixation rate, both well established indicators of primary photochemistry (Lavorell&Etienne,1977;Ogren,1990). Figure 8.8 shows the effect on chlorophyll fluorescence of nitrate addition to nitrogen-limited cultures. A substantial reduction in fluorescence oscillation occurred almost immediately with the addition of 0.1mM KNO_3 and is indicative of the sensitivity with which changes in the cell environment are reflected by the photosynthetic apparatus. A direct measurement of induced nitrogen-deficiency stress was precluded by methodological problems associated with removing nitrogen from the culture medium, without distorting the fluorescence pattern and thus rendering it meaningless.

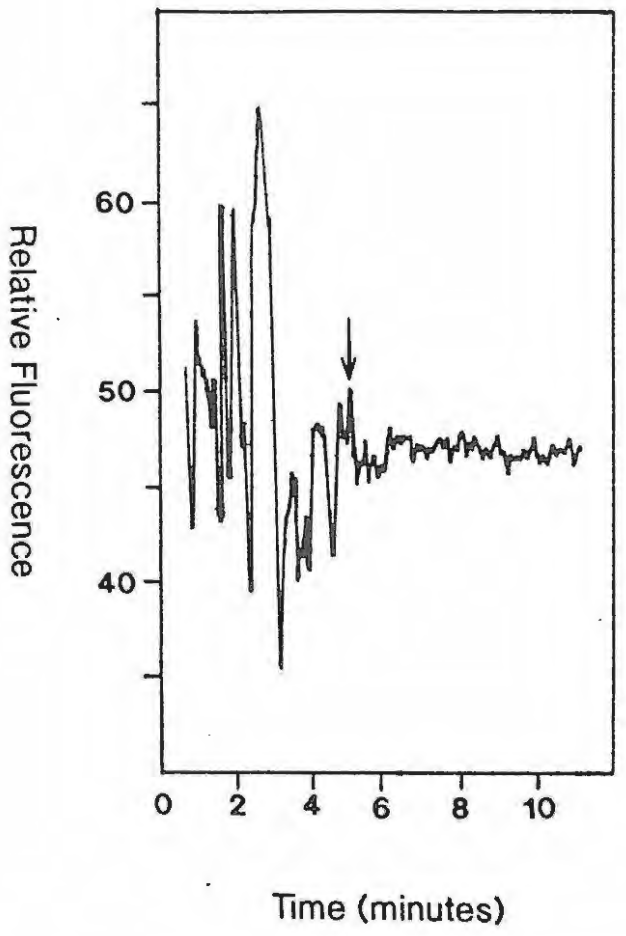


Figure 8.8 Damping of fluorescence oscillation in a nitrogen-deficient *D.salina* culture. Nitrate was added at a final concentration of 0.1mM KNO_3 (indicated by arrow).

Figure 8.9 records fluorescence changes on the addition of 0.1mM NH_4Cl to the culture medium. A rapid, temporary suppression of photosynthesis by ammonium has been previously demonstrated in *Dunaliella* (Turpin,1983). The effect is shown to be concentration-related and the culture sustains repeated shocking at the 0.1mM level while 5mM NH_4Cl produces an irreversible fluorescence peak (results not shown) associated with the permanent uncoupling of photosynthesis (Abeliovich,1986). Both levels of treatment show the consistent presence of an early fluorescence response pattern that appears within seconds of introducing the stress condition and lasts for approximately four minutes.

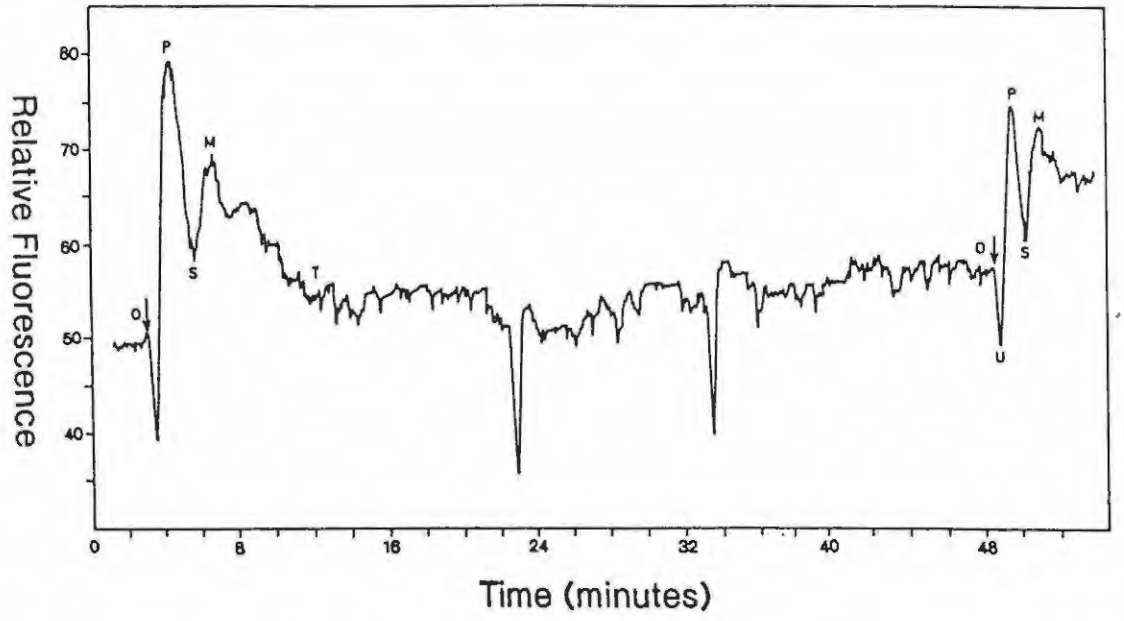


Figure 8.9 Fluorescence induction response in a *D.salina* culture at pH 7.6 with the addition of successive amounts of ammonium. The final concentration was 0.1mM NH_4Cl in each case. The signal response is identified by points marked O U P S M T and the ammonia addition by the arrows.

The fluorescence response pattern observed here is comparable with the previously described process of fluorescence induction known as the Kautsky effect (Walker,1987). The conventional notation O P S M T has been used to characterise the points in figure 8.9 with an additional point of quenched fluorescence, arbitrarily termed U, occurring between points 0 and P. The points I and D, which commonly occur in the first few seconds of fluorescence induction between points O and P, normally require oscilloscope measurement and were not observed in this study. A period of delay is inherent in the design of the continuous flow fluorometric apparatus used, allowing for mixing times in the reactor vessel and flow time to the fluorometer cell which precludes the measurement of the first seconds of fluorescence induction.

Despite the complexity and variability that is known to be associated with the fluorescence signal (Ogren,1990), an early response pattern, in some form, was found to be a consistent feature in each case of stress examined (figures 8.9 to 8.14). The response appeared almost immediately with the imposition of the particular stress, lasted between two and four minutes and was then followed by a subsequent response pattern which was more specific to the particular stress applied. This latter part of the fluorescence induction response is characterized by the point M, which has been associated with the termination of the induction of the carbon assimilation response following a dark-light shift (Walker,1987). Although the timing is appropriate, considering the carbon fixation results reported in figures 8.15 and 8.16, no evidence was acquired here to support this assumption. A settling down phase has been termed T.

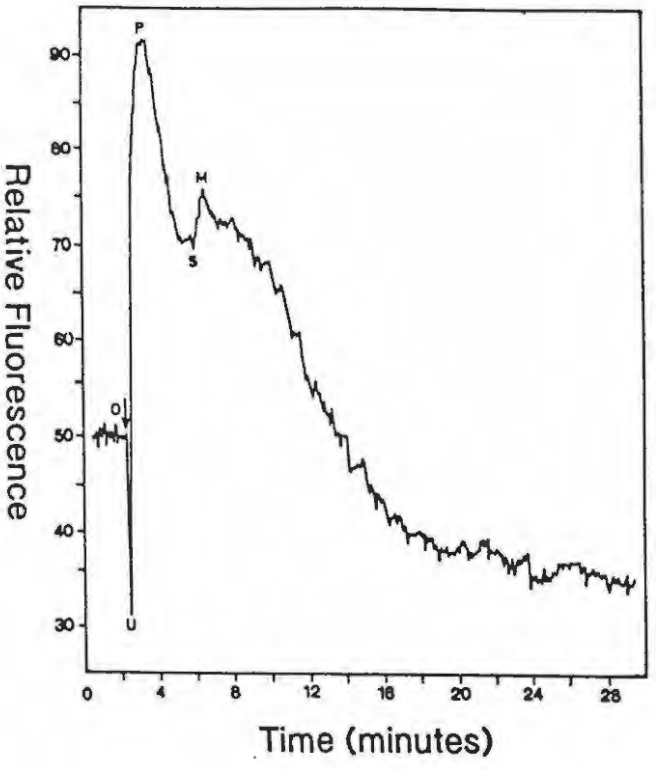


Figure 8.10 Fluorescence induction response in a *D.salina* culture at pH 9.1 with the addition of ammonium (indicated by arrow) to a final concentration of 0.1mM NH_4Cl.

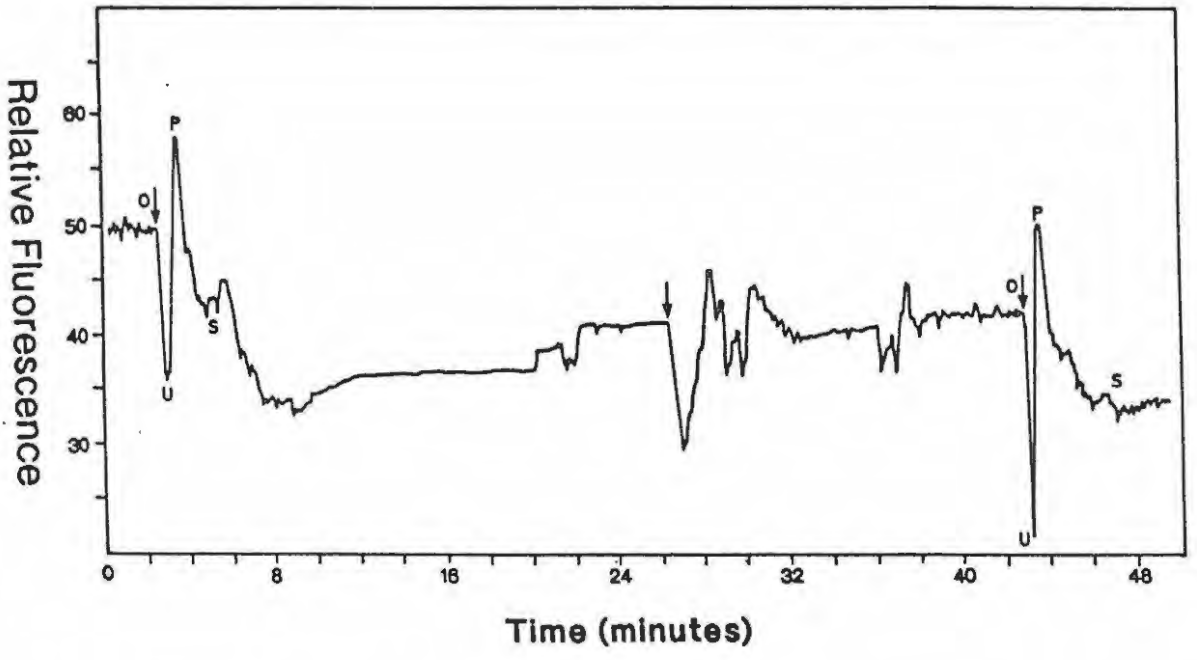


Figure 8.11 Fluorescence induction response in a *D.salina* culture at pH 7.6 with the addition of successive amounts of glycine (indicated by arrow). The final concentration was 1mM glycine and the signal response is identified by the points O U P S.

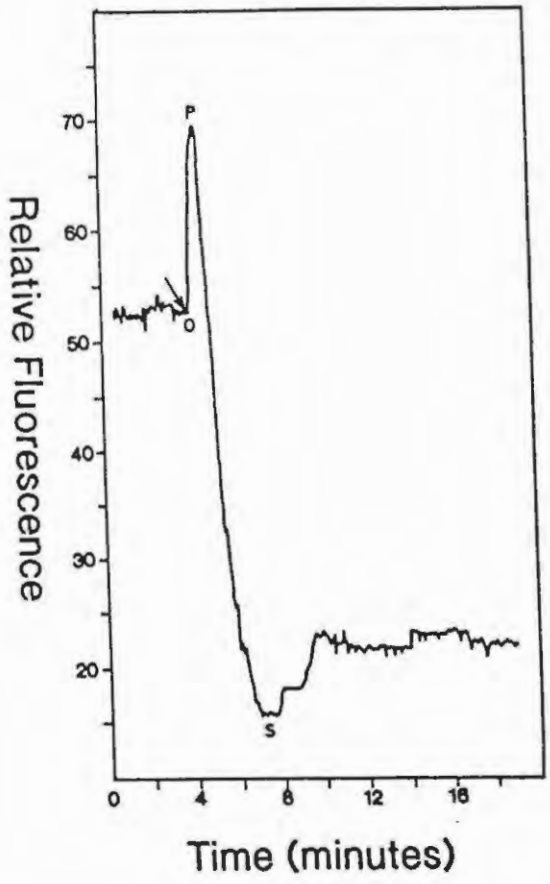


Figure 8.12 Fluorescence induction response in a culture of *D.salina* exposed to high light stress. The shift in illumination was from 147μmol.m^{-2}.sec^{-1} to 1500μmol.m^{-2}.sec^{-1}. Temperature was maintained constant at 30°C. The full response is identified by the points O P S.

The point U, indicating an initial fluorescence quench, was observed with the addition of NH_4Cl (figures 8.9,8.10), glycine (figure 8.11) and on a shift from near dark to medium light illumination of *D.salina* cultures. An interpretation of this point in figure 8.9 is consistent with a sudden demand for NAD(P)H and ATP required by the GS-GOGAT ammonium assimilation mechanism. High ammonium levels would activate both the glutamate dehydrogenase and glutamine/glutamate synthetase enzyme systems resulting possibly in an initial disproportionate demand for NAD(P)H, and a transient accumulation of glutamine (Danks *et al.*, 1983). Ammonium uptake, unlike nitrate, is not regulated by cellular nitrogen demand in the micro-algae (Kanda *et al.*,1990;Flynn&Fielder,1989) and a portion of that part present as NH_3 may diffuse directly across the membrane circumventing the uptake mechanism (Chevalier&de la Noue,1985).

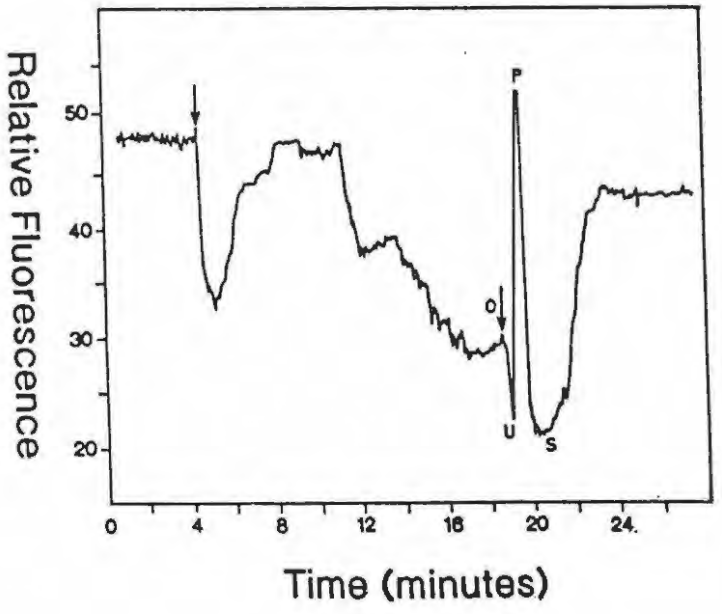


Figure 8.13 Fluorescence response in a *D.salina* culture subjected to successive light shifts. First a shift from medium to low light (147 to 20μmol.m^{-2}.sec^{-1}) then from a low light to near dark shift (20 to approximately 0μmol.m^{-2}.sec^{-1}) and finally to a shift back to medium intensity illumination (approximately 0 to 147μmol.m^{-2}.sec^{-1}). The timing of each shift is identified by an arrow and the signal response is identified by the points O U P S.

Both free ammonia and amines are known to be able to collapse the proton gradient in the chloroplast, acidifying the stroma and resulting in an uncoupling of photosynthesis (Danks *et al.*,1983;Abeliovich,1986). This would be expected to result in the relative alkalization of the cytoplasm.

Figure 8.10 shows the fluorescence profile where NH_4Cl is added at pH 9.1 where NH_3 is present as the predominant form (Abeliovich,1986). Compared to the addition at pH 7.6 (figure 8.9), the duration of the O U quench transient is reduced from 26 to 6 seconds and the U P S recovery response is extended from 108 to 156 seconds. The relative fluorescence value is then restored to well below starting steady state levels, which accords with the finding by Turpin (1983) that photosynthetic carbon fixation is enhanced immediately following the ammonia suppression effect. This suggests that increased carbon fixation is required to cope with NH_3

uptake. By contrast NH_4^+ addition (figure 8.9) resulted in a fluorescent steady state following the shock, which was higher than the initial value. This implies a coping mechanism that involves a diminished delivery of NAD(P)H and ATP by the photosynthetic apparatus. This is consistent with the regulation of the ammonium uptake mechanism.

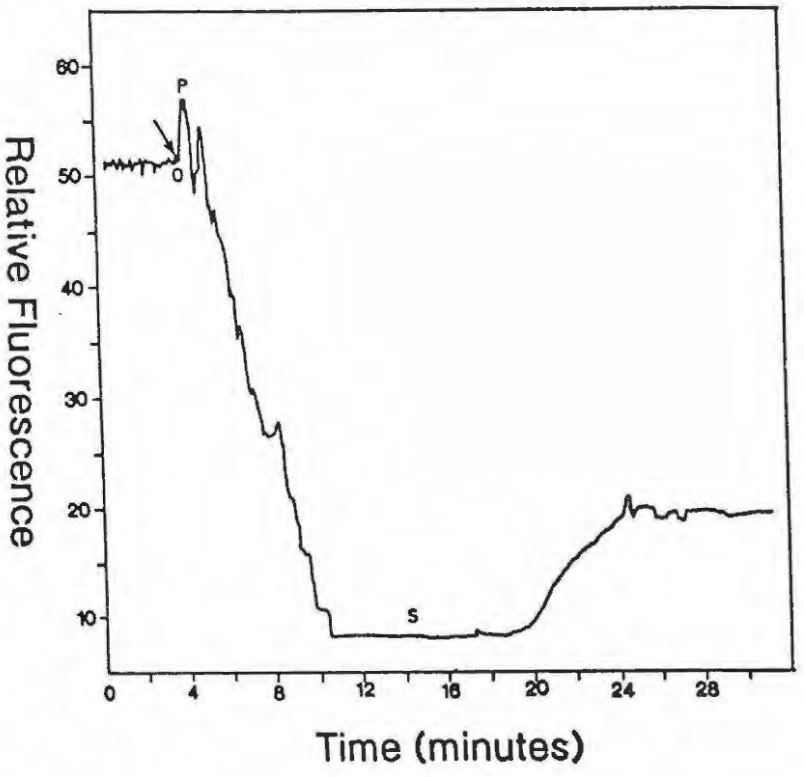


Figure 8.14 Fluorescence induction response in a *D.salina* culture subjected to a high salinity shock. Salt was added to give a final concentration of 3.5M NaCl. The signal response is identified by the points O P S.

These results, together with the reduction in fluorescence oscillation observed with the addition of KNO_3 (figure 8.8), suggest that the stress signal to the chloroplast, in the case of nitrogen-related stresses in *D.salina*, may be transduced via sudden changes in NAD(P)H and ATP requirements. The addition of nitrate to a nitrogen-limited culture would tend to saturate membrane-linked nitrate reductase enzyme binding, create a constant demand for NAD(P)H and thus level out the oscillations of fluorescence. The data is also consistent with an interpretation of the transient quench O U being due to a sudden demand for NAD(P)H where NH_4Cl is presented to the GS-GOGAT ammonium uptake system. Heineke *et al.* (1991) has defined NADPH/NADH redox transfers across the chloroplast membrane and Ogren (1988) has demonstrated the enhancement of stress effects by even slight decreases in the nitrogen status of Willow leaves.

In the case of the prolongation of the recovery phase U P S that was observed with NH_3 addition (figure 8.10), a link is suggested between the U P fluorescence peak and the uncoupling of photosynthesis. The U P peak is a common part of the stress signal also noted in the high light (figure 8.12) and the saline shock fluorescence induction responses (figure 8.14). The sharp quenching of fluorescence, observed in this study as P S, has also been correlated with redox changes across the chloroplast membrane which disturbs the alkalinity of the chloroplast relative to the cytoplasm of the cell. Bental *et al.* (1990) has demonstrated in *D.salina* that the alkalization of the cytoplasm is part of the initial response to salinity stress, signalling glycerol production. The pronounced fluorescence quenching that is characteristic of the hypersalinity stress response (figure 8.14) has been associated with the re-establishment of an additionally large ΔpH across the thylakoid membrane in *Dunaliella* (Gilmour *et al.*,1985;Canaani,1991). Yin *et al.* (1990), using fluorescent dyes, have demonstrated an equivalent alkalization of the chloroplast during fluorescence quenching following high light stress (see figure 8.12).

Within the photosynthetic apparatus itself, the rise in fluorescence O P may be linked to rapid reduction of Qa, the primary electron acceptor of photosystem 11 (PS II) which, in its oxidized form, is responsible for Qa quenching (Duysens&Sweers,1963), and a discharge of the proton gradient as the initial photosynthetic response to the stress. The subsequent fall in fluorescence PS may result from the re-oxidation of Qa and the commencement of Qe quenching (Krause *et al.*,1982) with the re-establishment of the proton gradient across the thylakoid membrane.

A further observation from the results of NH_4Cl (figure 8.9) and glycine (figure 8.11) effects on fluorescence was that the initial response could not be elicited again within 40 to 60 minutes of the first response, despite several intervening challenges. An O U-type quench is the only response to the addition of further aliquots of NH_4Cl or glycine during this period, and is followed by a rapid return to steady state levels of fluorescence. A similar time link for the restoration of the full fluorescence induction response could not be demonstrated for light and salinity stresses. These data suggest the presence of a sensitivity mechanism as defined by Trewavas (1991), and is further supported by differences in the response of carbon fixation to the various stresses imposed.

8.4.5. Photosynthetic Stress Response - Carbon Fixation.

The results recorded in figures 8.15 and 8.16 show changes in the carbon fixation rate on the imposition of salinity and combined salinity and nitrogen-stresses under conditions of high and low light. Both early and late responses were also apparent. The initial measurement was made 5 minutes after the imposition of the stress and shows a substantial fall in C fixation for each stress imposed, which is consistent with a temporary uncoupling of photosynthesis. The combinations of stresses exert a synergistic enhancement of the response. In each case the combinations of nitrogen and salinity exert the largest response which is consistent with the results recorded in figures 7.1 and 7.4. This emphasises the advantage of nitrogen-stress, and also supports the

observation of a nitrogen deficiency-induced enhancement of sensitivity to salinity stress (Ogren,1988). The results suggest the presence of an initial signal transduction mechanism that can, in addition, discriminate between the form and possibly also the intensity of stresses applied to the algal cell.

Figure 8.16 shows the longer term response where carbon fixation rates have been normalised to prestress starting values to enable a direct comparison by excluding the early response. High light stress alone resulted in a fall in carbon fixation at 6 hours which has returned to normal levels by 24 hours. This result is in contrast to the fluorescence quenching observed in figure 8.12 and suggests the use of either internally stored carbon or possibly also CO_2 recycling by photorespiration during this period.

The salinity and salinity plus nitrogen-stresses by contrast showed an enhanced fixation of carbon at 6 hours followed by a substantial decline at 24 hours. This response could be related to carbon storage and protein synthesis events preceding the onset of stationary phase conditions. In addition to an indication of early and late responses to stress, the carbon fixation results also show a clear difference between the responses to light and to the other forms of stress which has been previously noted (Sadka *et al.*,1991).

The results recorded in this study appear to show that a close relationship exists in *Dunaliella* at the physiological, biochemical and molecular levels between ABA and β-carotene accumulation on the one hand, and associated responses by the photosynthetic apparatus on the other.

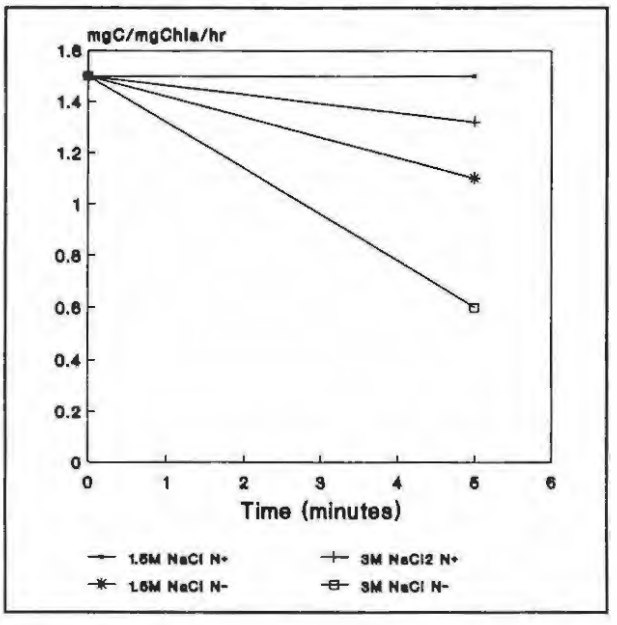


Figure 8.15 Early changes in carbon fixation rate. The reading was recorded after 5 minutes in response to increased illumination from 100-1500μmol.m^{-2}.sec^{-1}. Additional stresses imposed were nitrogen deficiency and nitrogen-deficiency plus hypersalinity.

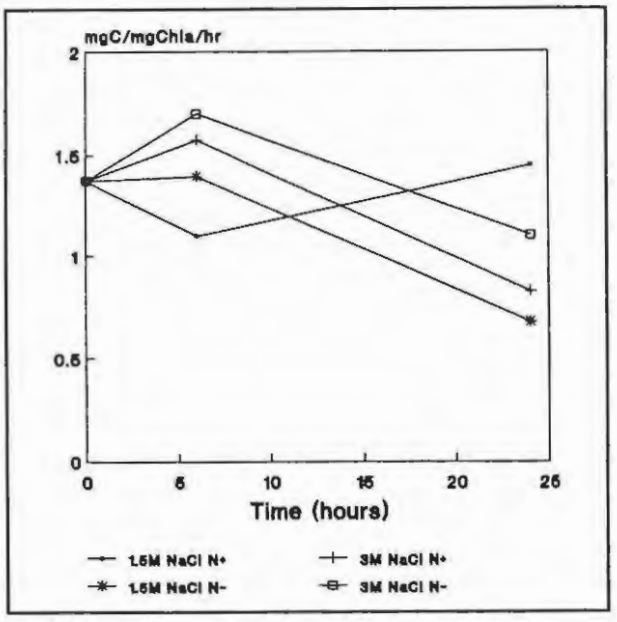


Figure 8.16 Later changes in carbon fixation rate in response to increased illumination. The reading was recorded at 6 and 25 hours with illumination increased from 100-1500μmol.m^{-2}.sec^{-1} (The 5 minute result has been excluded to allow a comparison of the longer term responses). Additional stresses imposed were nitrogen-deficiency and nitrogen-deficiency plus hypersalinity.

8.5. DISCUSSION.

The results of this study have confirmed the presence of ABA in *D.salina* and suggest it is produced in a biologically active form. The possibility that it could be a non-functional metabolic artifact, with a prospective evolutionary role, as speculated by Hirsch *et al.* (1989), seems to be discounted by tbe evidence provided here, which links its metabolism to the onset of stress conditions in *Dunaliella.* Changes observed in the photosynthetic apparatus of the alga confirm the actual induction of stress effects by the various manipulations of its environment.

8.5.1. Metabolism.

The results also provide evidence which suggests an interrelationship between ABA production and the induction of β-carotene production in response to hypersalinity stress. Two stages of β-carotene accumulation have been demonstrated. The first stage commences immediately after exposure to hypersalinity and coincides with increased endogenous ABA levels. This stage lasts for approximately 24 hours, after which the β-carotene content remains constant until the second stage of accumulation begins. By comparison, cellular levels of ABA remained constant, whereas levels of ABA in the culture medium increased almost linearly over the remainder of the 5 day incubation period. These data are in keeping with earlier reports concerning the effects of salinity and high-light intensity on β-carotene accumulation by cells of *D. salina* (Borowitzka *et al.*,1990;Lers *et al.*,1990) and suggest an interrelationship between β-carotene and ABA production.

Evidence has been obtained to suggest that ABA is derived from the oxo-carotenoid, 9-*cis* neoxanthin (Parry *et al.*,1990). The over-production of carotenoid by cells of *D.salina* in response to stress (Ben-Amotz&Avron,1983,1989b;Borowitzka *et al.*,1990;Lers *et al.*,1990) might therefore be regulated, at least in part, by the synthesis of ABA and the subsequent partitioning of ABA and some of its metabolic products into the culture medium. In higher plant tissues, ABA has been shown to induce its own conversion to PA (Uknes&Ho,1984). The results presented in this investigation show that amounts of both PA and ABA isolated from the culture medium of salt-stressed cells were higher than in the medium from nonstressed cells. This observation supports the enhanced catabolism of ABA in response to stress.

Results from studies using labelled precursors and inhibitors of both transcription and translation suggest that the induction of ABA production occurs by *de novo* synthesis. Similarly, the induction of β-carotene accumulation in response to stress is known to occur by *de novo* synthesis (Sadka *et al.*,1989). Furthermore, several stress-induced proteins have been isolated which could fulfil a carotenogenic function (Lers *et al.*,1990), possibly by influencing the metabolism of ABA. In addition, norflurazon, which is known to inhibit ABA biosynthesis in higher plants (Creelman,1989), also prevents the accumulation of β-carotene in *Dunaliella* (Ben-Amotz *et al.*,1987;Ben-Amotz&Avron,1989b), further supporting an interrelationship between ABA production and β-carotene accumulation in cells of *D.salina*. It has been suggested that the early stage of β-carotene accumulation is mediated by the activation of gene(s) encoding enzyme(s) responsible for β-carotene synthesis (Lers *et al.*,1990). Results from the present study suggest that enhanced ABA production and catabolism during the early stage of β-carotene accumulation might shift the chemical equilibrium to favour carbon flux through the isoprenoid pathway, the end result being enhanced carotenoid biosynthesis, possibly mediated by gene activation and/or alterations in enzyme synthesis and/or activity.

The second stage of β-carotene accumulation may result from continued ABA synthesis, ABA catabolism, and subsequent partitioning of ABA and its acidic products into the culture medium. Cells do not appear to accumulate significant quantities of ABA during this stage, and label from supplied (R,S)-[2-^{14}C]ABA was not incorporated by cells of *D.salina* (data not shown). This accumulated information strongly suggests an interrelationship between ABA production by, and β-carotene accumulation in salt-stressed cells of *D.salina*. Thus, increased production of ABA and the enhanced conversion of ABA to PA and subsequent release of these compounds into the culture medium could function to initiate the accumulation of carotenoid in particular, β-carotene.

8.5.2. Photosynthesis.

The form of the response of the photosynthetic apparatus to salinity stress found in this study suggests the presence of a signalling stage that precedes the already identified β-carotene accumulation stages. Available evidence links this signal with pH changes in the chloroplast. Enhanced pH changes across the thylakoid membrane are considered to be responsible for the substantial chlorophyll quenching effect that follows the

imposition of a stress (Gilmour *et al.*,1985;Canaani,1986,1991), and transient alkalization of the chloroplast has been demonstrated in response to a dark-light shift by Yin *et al.* (1990) with the use of fluorescent dyes.

The common features of the initial fluorescence signal observed for the various stress conditions, including light, suggest the validity of a general interpretation implicating a sudden redox change across the thylakoid membrane and the relative acidification of the chloroplast stroma as the initial part of a common stress signalling mechanism involving the photosynthetic apparatus.

8.5.3. The Signal.

Based on this premise, a speculative role for ABA can be suggested in the transduction of the primary stress signal itself. The compartmentalization of ABA has been demonstrated in higher plant cells by Heilman *et al.* (1980), and Kaiser *et al.* (1985) where the acidic permeating form of ABA becomes trapped in alkaline compartments according to Henderson - Hasselbalch equilibria. More than 80% of the endogenous ABA occurs in the actively photosynthesising chloroplast (Heilman *et al.*,1980), which is alkaline relative to the cytoplasm (Yin *et al.*,1990). A sudden acidification of the chloroplast, reflected as a peak of increased fluorescence, could cause a partition of endogenous stores of ABA to the cytoplasm. Kaiser *et al.* (1985) has shown for higher plants that ABA leaves the chloroplast as it becomes acidic on transfer to the dark. The subsequent sharp fluorescence quenching response which Canaani (1991) has shown in *Dunaliella* to be associated with the re-establishment of an additionally large ΔpH across the thylakoid membrane would indicate the return of ABA to the chloroplast. However, the brief surge of ABA to the cytoplasm could be sufficient to facilitate binding to cytoplasmic membrane receptor sites (Owen&Napier,1988) and set off a cascade of responses associated with stress signalling. This response would not require an increase in endogenous levels of ABA, which concurs with the observation that a change in conductance precedes ABA accumulation in waterstressed leaves (Zeevaart&Creelman,1988).

A substantial body of evidence has been acquired, in higher plant systems, relating ABA to such a signalling mechanism and Creelman (1989) has reviewed evidence supporting both a fast and a slow response to ABA in higher plants. The fast response which lasts only a few minutes is characterized by effects on photosynthesis (Zeevart&Creelman,1988;Kirst,1989;Ben-Amotz *et al.*,1989), respiration (Owen&Napier,1990), membrane permeability (Stillwell&Hester,1984;Stillwell *et al.*,1987) and increased conductance of phospholipid bilayers and liposomal membranes for cations. Owen and Napier (1988) have reviewed the role of ABA in transducing primary stimuli into physiological responses via calcium agonism in a range of eucaryotes. ABA appears to enhance the permeability of the cytoplasmic membrane to calcium, the influx of which could be responsible for initiating a classic hormonal cascade of intracellular responses involving the calcium binding protein calmodulin. The calcium-calmodulin complex is thought to modulate enzyme activity and thereby elicit a physiological response. Cytoplasmic calcium has also been implicated in the activation of protein kinase C, part of a signalling mechanism that involves the turnover of cytoplasmic membrane inositol phospholipids. Einspahr and Thompson

(1990) have reviewed the evidence for the involvement of inositol phospholipids in transmembrane signalling in plants and also with specific reference to *D.salina* (Einspahr *et al.*,1988a,b,1989).

Ultrastructural changes to the cytoplasmic membrane of *Dunaliella* concurrent with the application of stress conditions have been demonstrated by Maeda and Thompson (1986) and Einspahr *et al.* (1988a,b) and also reported in chapter 6 of this study.

A role for cytoplasmic alkalization in triggering glycerol production in the osmoregulation response to hypersalinity stress has been demonstrated by Bental *et al.* (1990). Oren-Shamir *et al.* (1989) and Chitlaru and Pick (1989) have demonstrated a salinity stress-induced hyperpolarization of the cytoplasmic membrane which returns to a resting potential in about 30 minutes after the shock and involves the presence of a plasma membrane H^+-ATPase. These systems are considered to be the major generators of electrical gradients across the plasma membrane in higher plants (Oren-Shamir *et al.*,1989). ABA binding sites have been demonstrated on the plasma membrane of guard cells (Hemberg&Weiler,1984) and it has also been shown that ABA is able to block an H^+/K^+ antiporter in the plasma membrane (Rascke,1987).

Hirsch *et al.* (1989) had speculated that ABA in the algae might act on the plasma membrane affecting the transport of solutes leading to transient or permanent changes in cellular solute distribution and thereby producing a hormonal signal.

The slow, longer term effects of ABA are linked to its implication in transcriptional and translational events (Stillwell&Hester,1984;Singh *et al.*,1989). ABA is known to be able to induce its own set of proteins (Ucknes&Ho,1984;Singh *et al.*,1987;Creelman,1989) and it has been linked to the regulation of storage protein synthesis in higher plants (Zeevaart&Creelman,1988). Both osmotic stress and exogenously applied ABA have been sbown to produce similar effects in promoting mRNA and protein synthesis (Finkelstein *et al.*,1985).

Sadka *et al.* (1991) have demonstrated the induction of a 150kD cell surface glycoprotein in hypersaline-stressed cells of *D.salina*. Glycoproteins embedded in the plasma membrane could be associated with the glycocalyx type cell envelope demonstrated ultrastructurally by Oliviera *et al.* (1980). The present study has confirmed an increase in the glycocalyx layer in response to stress conditions (see figure 6.7) and that the layer may have membrane-like properties of its own. The function of membrane glycoproteins bave been linked to the operation of energy-dependant efflux pumps (Endicott&Ling,1989).

Lers *et al.* (1990) has demonstrated the induction of a 55kD protein in response to high light stress. *De novo* protein synthesis has also been shown to be involved in state transitions between PSII and PSI in *Dunaliella* in response to changes in light regime (Sukenik *et al.*,1990).

8.5.4. Sensitivity.

Besides the similarities that are apparent in the physiological responses of *D.salina* to various stress stimuli, the results of this study also show differences in the initial signalling stage of the stress response which could be significant.

The various stresses exerted different effects on carbon fixation, which has been shown to occur over the same time period as the initial signalling stage (see figure 8.15). This suggests an ability by the system to mount a variable response depending on the form and the intensity of the stress imposed. ABA has been shown to influence photosynthesis via a direct effect on carbon fixation (Raschke&Hedrich,1985;Ward&Bunce,1987). Kirst (1989) has noted that both photosynthesis and respiration are inhibited in the micro-algae under extreme hypo- and hypersalinity stresses with a recovery established within minutes.

This evidence suggests the operation of a sensitivity mechanism in *Dunaliella* of a kind demonstrated by Trewavas and others in higher plants (Trewavas,1991). The observation that under certain conditions the initial fluorescence signal could not be elicited again within approximately 40-60 minutes is in agreement with the finding by Oren-Shamir *et al.* (1990) that membrane hyperpolarization in salt stressed cells returns to resting potential within about 30 minutes. The ABA production peak at 30 minutes (figure 8.3) is in general agreement with this finding and its rapid excretion and conversion to PA after this point could possibly be correlated with a freeing of ABA membrane binding sites.

A further level of control is suggested by the results reported by Singh *et al.* (1987,1989) for the production of the 26kD salinity stress protein, osmotin, in tobacco cells. The encoding of the specific mRNA has been shown to be induced by ABA but the accumulation of the protein depends on the actual presence of a low water potential. This seems to imply that the signal and the response could also be seen as two clearly defined events, where the initiation of transcription depends on elevated ABA levels, while *de novo* protein synthesis is at least partly regulated by the continued presence of the particular stress condition.

8.5.6. Stages of Response.

It should be emphasised that aspects of the above discussion are based on a speculative interpretation of available data. The common feature of β-carotene accumulation as a stress response has already been demonstrated (Ben-Amotz,1986,1987) and has been confirmed in this study. This suggests the possibility, in theory at least, of a generalized role for ABA in the stress physiology of *D.salina*.

Assuming the validity of the conclusions relating ABA to β-carotene accumulation in response to salinity stress and the presence of a signalling process which forms part of a sensitivity control mechanism, it may be possible to suggest a comprehensive view of the whole stress response event that results in pigment accumulation. It could function in four more or less clearly defined stages.

1. Stage 1. The Signal.

   The imposition of a stress is reflected in photosynthetic changes by some means, which could involve a transient change in the demand for NAD(P)H that results in a short-lived acidification of the chloroplast. An ABA surge to the cytoplasm initiates a cascade of responses including possibly phosphoinositide changes, a Ca^{2+} flux and a calmodulin-modulated response. This stage would not require a change in endogenous levels of ABA and is completed within a few minutes.

2. Stage 2. ABA Accumulation.

   ABA starts to accumulate in the cell with a peak in concentration at 30 minutes, and exerting an effect on the induction of specific proteins. Although evidence is scanty, it may be possible to suggest that moving from stage 1 to stage 2 requires the continued presence of the stress event, without which the initial response process may be aborted. The accumulation of ABA may reflect this transition in the development of the response. The excretion of ABA and its degradation to PA are part of a continuing metabolic process that is in some way involved with the accumulation of β-carotene.

3. Stage 3. Early β-carotene Accumulation.

   An early phase of β-carotene accumulation that is concluded within 24 hours has been demonstrated for salinity stress in this study and for light stress. In the case of light stress this has been suggested to serve a photoprotective function.

4. Stage 4. Late β-carotene Accumulation.

   The onset of stationary growth phase, whether induced by light or by other stress conditions, and which commonly occurs within 2-4 days, is associated with a further stage of β-carotene accumulation. This accounts for the largest part of the final cellular concentration of β-carotene. ABA is produced continuously and excreted by the cell throughout this stage.

CONCLUDING REMARKS.

Substantially more evidence will be required to confirm the speculative proposal outlined above. This should include an evaluation of changes in the levels of other pigments associated with the ABA response, in order to establish the metabolic mechanism in which it may be involved in the regulation of carotenoid biosynthesis. The presence of ABA changes similar to those observed for salinity stress will need to be demonstrated for other stress stimuli. The partitioning of endogenous levels of ABA in response to stress and the demonstration of cytoplasmic membrane ABA binding sites in *Dunaliella* would also be required to confirm the presence of a sensitivity stress-control mechanism.

Nevertheless, the empirical observations associated with an early ABA peak, with subsequent β-carotene accumulation occurring after some days, does provide an encouraging prospect for developing a physiological probe for predicting the efficacy of a particular manipulation strategy applied in the mass culture biotechnology of producing β-carotene from *D.salina*.

PART C:

PRACTICAL APPLICATION

The theoretical advantages of the multistage process for enhancing system yields in *D.salina* β-carotene production, and in overcoming nitrogen inhibition with the use of organic effluent media have been noted. The uptake and utilization of organic nitrogen by *D.salina* has been demonstrated. The use of the hormonal regulation of the stress-induced metabolite accumulated as a physiological probe for manipulating a separate stress unit of protein has also been noted and discussed. The process, however, also requires effective control over the transfer of algal biomass produced, from a cell growth to a stress unit, where metabolite accumulation would take place.

The cell separation technology required to accomplish this transfer presents a potential technical bottleneck which could challenge the feasibility of the entire concept. It is generally accepted that effective separation techniques for micro-algae still presents one of the major limitations in the development of algal biotechnology (Richmond,1986c;Mohn,1980,1988;Ben-Amotz&Avron,1989a) and represents one of the largest cost centres in algal production (Abeliovich,1986). Furthermore, little attention appears to have been given to the harvesting of micro-algae in a viable form. This is a prerequisite for practicalities of the successful operation of the subsequent stages of the process which require continued photosynthesis.

Novel processes in biotechnology are faced with an inherent requirement to demonstrate practicality, together with theoretical feasibility, if they are to avoid the accusation of research dilettantism, and result in possibly unwarranted down-stream development expenditure. Hacking (1986) has warned that many failures in biotechnology development can be linked to an overemphasis on the study of the biological step without a satisfactory consideration of the wider practical and economic implications. If that lesson is to be taken seriously, progress in this project will need to be demonstrated at both the fundamental and applied levels.

RESEARCH OBJECTIVES.

The broad objective of the final part of this investigation was to demonstrate a practical application of the fundamental, theoretical and speculative results acquired in the earlier parts of the study. The end result of the technical study, identified at the outset, should be the formulation of a process design which incorporates both biological and technical requirements, and can serve as a basis for subsequent scale-up evaluation studies. In order to tackle this objective it was necessary to first demonstrate the availability of an effective cell separation process that could accomplish the biomass and media transfers, which are essential prerequisites to the successful operation of the multistage concept.

CHAPTER 9

AN EVALUATION OF MICRO-ALGAL CELL SEPARATION

Summary.

Cross-flow ultrafiltration is shown to provide an enabling technology for the operation of the multistage process. This system of cell separation was optimised to produce cell concentrates of *D.salina* in a viable form. When operated in the diafiltration mode, the nutrient status of the concentrates produced can be determined, allowing a more precise control over the function of the stress unit function. Growth medium can he removed as a virtually sterile permeate which can be recycled enabling a form of control over the problems of competition and predation in the growth unit. A scale-up application of the system evaluated in an industrial environment is reported.

9.1. INTRODUCTION.

The technology for separating algal biomass from dilute suspensions of cells has been extensively reviewed (Golueke&Oswald,1965;Benemann *et al.*,1977,1980,1987;Mohn,1980,1988;Richmond,1986a;Ben-Amotz& Avron,1989a). While the separation of pure algal cultures has received attention, most reports relate to waste grown biomass from HROP systems treating municipal effluents, removed to comply with environmental discharge standards.

It has been well recognised since the early developments in micro-algal biotechnology (Tamiya,1957) that the feasibility of large-scale production will depend substantially on effective technology for harvesting and concentrating dilute suspensions of cells (Mohn,1980). Despite significant advances in other areas of algal production, the economics and efficiency of separation techniques still represents a major limiting factor in large-scale commercialization (Richmond,1986b;Ben-Amotz&Avron,1989a). The practicality of novel developments in algal culture technology will, as a result, depend to some degree on the simultaneous addressing of the cell separation issue.

While the larger filamentous algae such as *Spirulina* can be successfully and inexpensively separated by various screening techniques (Mohn,1980;Hensman,1985;Mitchell,1986), the unicellular micro-algae present a particularly difficult problem as a result of their small size, low density and inter-particle repulsion due to a strong negative surface charge (Oliviera *et al.*,1980). The separation of the fragile *Dunaliella* presents additional constraints where the absence of a rigid cell wall can result in rupture and the loss of intracellular products during mechanical harvesting processes (Naghavi&Malone,1986). A factor which has not been previously

evaluated is the harvesting of cells in an intact, viable and actively metabolising form, essential to the multistage processing concept.

A wide range of cell separation techniques have been the subject of literature evaluation including flocculation (Golueke&Oswald,1965;Middlebrooks *et al.*,1974;Soeder,1976,1978;Benemann *et al.*,1980;Lincoln&Hill,1980;Moraine *et al.*,1980;Sandbank&Hepher,1980;Venkataraman,1980;Klut *et al.*,1983;Lincoln&Koopman,1986;Sridhar *et al.*,1987), adsorption (Curtain&Snook,1982,1985) and phototaxis (Toha *et al.*,1990). An illuminated plate system has been patented by Ben-Amotz and Avron (1980). Centrifugation and filtration have received considerable attention and will be reviewed here.

9.1.1. Centrifugation.

Centrifugation is possibly the most widely used technique for the harvesting of the micro-algae in large-scale defined-medium culture operations (Mohn,1980,1988;Mackay,1988). A range of centrifuges have been evaluated including plate and nozzle separators operated in continuous automated discharge and batch modes. Cultures can be typically concentrated to 15-20% solids dry weight (SDW).

The advantage of centrifugation is that it can be relatively easily incorporated into engineering design and the density of the final concentrate can be adjusted over a wide range to optimise downstream processing. The system can be used to concentrate any type of micro-algae and the equipment is easily cleaned and sterilized. The product is also usually free of additional chemical contamination.

The major drawback is that centrifugation is also one of the most costly of available cell separation processes, both in terms of capital investment and operational energy costs (Mohn,1988). Tapie and Barnard (1988) estimate the cost of centrifugation harvesting in open pond algal culture systems at about 20% of operating costs and 10% of total depreciable capital. Salinity corrosion with halophilic systems can result in a considerable reduction in the operational life of the plant. Automated discharge results in a dilution of the concentrate while manual desludging of batch-type bowl centrifuges is labour intensive. Damage to fragile cells can be significant (Borowitzka *et al.*,1986), especially in continuous discharge centrifuges and the recovery of viable cultures in satisfactory amounts for the operation of a multistage process is not likely to prove feasible using centrifugation.

The centrifugation process removes all particles of the size and density of the particular algae for which the system has been designed, leaving the smaller suspended particles in the medium. This can result, with the recycle of the growth medium, in the selective accumulation of smaller particles including contaminating algae and other micro-organisms such as fungi, halophilic bacteria and their spores (Moraine *et al.*,1980;Ben-Amotz&Avron,1989a). Reduced light penetration due to the accumulation of dust and dirt particles in the growth medium may be an important contributing factor to the sub-optimum yields reported for open pond algal cultures (Richmond,1988b).

9.1.2. Filtration.

The economics of filtration, as a separation process used in biotechnology, compares favourably to most other available techniques (Hacking,1986). In the field of micro-algal separation a wide range of filtration systems have been evaluated including sand filtration (Backes,1987;Naghavi&Malone,1986), simple cloth type filters for rural conditions (Becker&Venkataraman,1982), micro-screening (Mitchell,1986) and a range of filter presses, belt filters, pressure suction filters, vacuum drum filters and diaphragm presses with various pre-coatings (Mohn,1980,1988). In general it has been found for the unicellular micro-algae like *Dunaliella* that filtration, especially where pressure is applied, results in damage to cells or presents desludging problems and low throughput rates.

Cross-flow (tangential flow) filtration (CFF) using either microfiltration (CFMF) or ultrafiltration (CFUF) membrane systems is a type of filtration that has not received adequate attention in the various algal separation evaluation studies reported in the literature. Since the development of this technique in the early 1960s by Sourinjan and Loeb (Tutunjian,1985), it has been used in a wide range of separation applications including removal of suspended solids from sewage (Bindoff *et al.*,1987) organics from river water (Treffry-Goatly,1987), the treatment of tannery effluents (Neytzel-de Wilde *et al.*,1987) and the clarification of fruit juices and removal of whey solids from milk.

At an early stage, biomass harvesting applications were also investigated by Michaels in 1968 (In:Le&Howell,1985) and Blatt *et al.* (1970), and cross-flow filtration has subsequently been applied with considerable success in a wide range of cell culture, separation and concentration processes in biotechnology (Tutunjian,1985;Bailey *et al.*,1990). This includes the concentration of fragile mammalian cells, continuous fermentation cell recycle systems using yeasts and other micro-organisms (Ohleyer *et al.*,1985;Hoffman *et al.*,1987;Cheryan&Mehaia,1983,1984) and anaerobic digestion solids recycle systems (ADUF), (Bindoff,1987;Ross&Barnard,1989).

Water clarification involving the separation of algal cells has also been investigated (Kovalenko,1978;WRC Fish Water Flats project - report pending). A method for the recovery of *Dunaliella* in diatomaceous earth, used as a cross-flow microfiltration filter aid, was patented by Ruane (1974a,b). The algae were collected in the diatomaceous earth, removed and β-carotene recovered by solvent extraction. Ben-Amotz and Avron (1983,1989a) report the evaluation of a cross-flow filtration process for the separation of *Dunaliella* but do not specify whether this was microfiltration or ultrafiltration. They found, however, that continuous operation was not possible with the filter blocking, possibly due to cell damage, shortly after the application of filtration pressure. They discarded further use of the process. This result is surprising given the successful filtration of equally fragile cell systems.

The principles of ultrafiltration have been reviewed by Le and Howell (1985). Membrane fouling is a disadvantage of the system and current theories explaining gel polarization and membrane clogging effects, and techniques used to overcome them have been reviewed by Tutunjian (1985). A gradual flux reduction over the period of operation is anticipated and the effectiveness of flux regeneration, using a variety of cleaning cycles, is used as an indication of the suitability of an application.

The use of cross-flow filtration as a diafiltration or cell washing process has been reviewed by Gabler (1985). In this process, combining the effects of dialysis and filtration, solutes are removed and particulates retained by the addition of fresh medium. This washing effect occurs at a constant volume and the product concentration does not change. The degree of filter retention of the solute determines the rejection coefficient of the system and hence the volume of diafiltration wash required to produce a desired rate of removal. This can be performed at relatively low solids levels in a continuous feed and bleed mode. Up to 98% liquid replacement within 4 volume cycles is commonly achieved.

Mohn (1988) has pointed out that the degree to which solids are concentrated should not alone be used to evaluate various competing systems. Where liquid handling of the product is envisaged for downstream processing i.e. spray drying, UHT treatment or solvent extraction, cell concentrates of around 8-10% are most appropriate.

In recent years membrane separation has developed into a robust industrial process and given its numerous theoretical advantages as a potential algal cell separation technique, it is surprising that it has not found wider application in mass culture algal biotechnology.

This chapter has attempted to address the rather surprising exclusion of CFUF from the substantial literature that has appeared on the separation of the micro-algae. It must also be borne in mind that industrial know-how is seldom published and that this comment relates to information available in the public domain.

The following advantages of CFF have been identified by Le and Atkinson (1985):

1. The process can be optimized to avoid excessive cell damage;
2. It is not dependant on density differences and no phase separation is required;
3. It can be readily engineered in continuous or batch modes and is easily scaled up by the addition of parallel units;
4. It is easily cleaned and sterilized;
5. Cell concentrates can he produced over the same wide range as centrifugation ie. up to 15% SDW.

9.2. RESEARCH OBJECTIVES.

1. To evaluate cross-flow filtration as a separation process to produce cell concentrates in a viable form and that can be feasibly engineered into a large-scale algal production facility;
2. To be able to recycle growth medium free of contaminating micro-organisms, dirt particles etc., thus enabling cell growth at lower controlling salinity levels;
3. To be able to wash cell concentrates for the removal of growth medium, especially where nitrogenous effluents are used and thus control the stress induction of metabolite production in multistage processing.

9.3. MATERIALS AND METHODS.

9.3.1. Filtration Unit.

The function and performance of the WRC cross-flow filtration semi-technical scale pilot plant (figure 9.1) was optimized before the commencement of the cell separation aspects of the experimental programme. Requirements for the optimization and scale-up of ultrafiltration processes has been reviewed by Tutunjian (1985) and this process is recorded in the results. *D. salina* cultures were grown in 20L glass flasks and in $1m^2$ open carousel paddle ponds (volume 200L). Filtration function was evaluated using cell counts and growth performance subsequent to filtration. Cell count methods have been previously described. Algal solids were determined gravimetrically. Analytical measurements were carried out according to APHA Standard Methods (1980) and as previously described.

9.3.2. Cross-Flow Microfiltration (CFMF).

A 4mm woven nylon hose (12mm diameter, $0.151m^2$ surface area) was fitted to the filter plant, 5L tap water (salinity appropriately adjusted - usually to 1.5M NaCl) was added to tank T1 (figure 9.1) together with 15g diatomaceous earth. Pumping was initiated and the system run until pressures stabilized, commonly at P-inlet = 260kPa and P-outlet = 150kPa (measured across points AB in figure 9.1), indicating appropriate deposition of the dynamic filter membrane. Once steady state conditions had been reached the make-up volume was reduced to approximately 2L when the culture or effluent to be treated was added. Permeate flow was measured at V5 (or V4 in the recycle mode) and the permeate flux calculated as $L.m^{-2}.hour^{-1}$.

9.3.3. Cross-Flow Ultrafiltration (CFUF).

A 9mm diameter polyethersulfone membrane tubular ultrafiltration unit (80 000 MW cut-off) (Membratek) was connected to the filter pilot plant at points A and B (figure 9.1). Liquids to be filtered were added to tank T1 and operational pressures and permeate flow rates recorded. The flux rate was calculated as above.

9.3.4. Diafiltration.

The tubular CFUF system was used to perform the initial cell concentration as described above. The concentration and washing of both *Dunaliella* cells in growth medium and the protein in Curers Brine Effluent were used to evaluate the process.

D. salina ($40x10^4$ cells.mL^{-1}) in an ASW stress medium (3M NaCl) were first concentrated to about 0.5% solids by CFUF and then, without interrupting the concentration cycle, a wash of 1.5M NaCl ASW was added until salinity levels returned to 1.5M NaCl. Cell damage was assessed microscopically as counts of cells remaining viable 24 hours after the process.

Curers Brine Effluent was concentrated by CFUF as described and then salts removed by diafiltration with the addition of tap water yielding a washed protein concentrate. Process efficiency was determined by analysis of raw feed, permeate and concentrate using methods already described. The quality of permeate produced was also used as an indication of the suitability of the CFUF process for clarifying the effluent preparatory to its use as a growth medium for the culture of *D.salina*.

9.3.5. Electron Microscopy.

Methods for the preparation and SEM examination of freeze fractured specimens were as previously described.

9.4. RESULTS.

Cross-flow filtration using both micro- and ultrafiltration membrane systems were evaluated using a semi-technical scale pilot plant filter rig developed for the Water Research Commission by the Pollution Research Group, Natal University (figure 9.1). This unit had originally been designed for woven hose dynamic membrane microfiltration studies on industrial effluents. A process of optimization was undertaken to adapt the design and operating procedures of the rig to function in either micro- or ultrafiltration modes.

Centrifugal type pumps were shown to cause complete shattering of cells over a short period of time. A positive displacement screw type pump (Mono) was found to give the best result. Feed velocity is controlled across valve V2 and inlet pressure across valve V3 which enables the control of filtration flux rates. The sudden pressure drop across these valves was found to be responsible for a rapid cell loss due to shattering. To overcome this problem a variable speed electric motor was fitted to the rig which enabled filtration pressure, and thus also flux rate, to be regulated by pump speed. A lowering of operational pump speeds to 150-200rpm and the location of the filter discharge pipe C below the feed tank liquid level also showed a significant improvement. The adapted system was able to circulate cultures of *D.salina* for prolonged periods of up to 12 hours with virtually no cell damage.

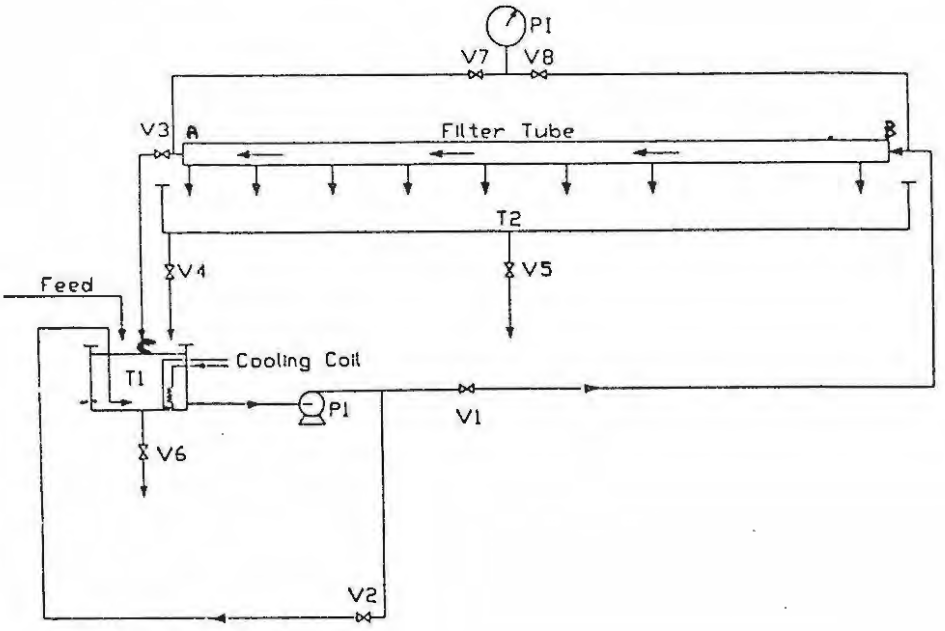


Figure 9.1 Schematic diagram of the semi-technical scale pilot plant cross flow filtration rig adapted to operate in micro- or ultrafiltration modes. (T1 = holding tank, T2 = permeate catch tray, P = pump, V = valves and P1 = pressure gauge.

9.4.1. Cross-Flow Microfiltration (CFMF).

An evaluation of cross-flow microfiltration for producing cell concentrates from *D.salina* cultures was undertaken. The microfiltration system, with diatomaceous earth as a filter pre-coat on a 4m woven teflon hose, was initialised as described under "Methods". The results are reported in figures 9.2, 9.3 and table 9.1.

Cell numbers showed a rapid decline at the start of filtration accompanied by a sharp fall in the flux rate and the accumulation of a green foam in the tank due to cell shattering. Flux rate showed a characteristic levelling but the attrition of cells, reflected as an almost constant cell number against a declining volume, continues throughout the process. Three hours after starting cell numbers had fallen by 49%. After a certain period of apparent steady state, there was a sudden complete loss of cells accompanied by a further fall in the flux rate.

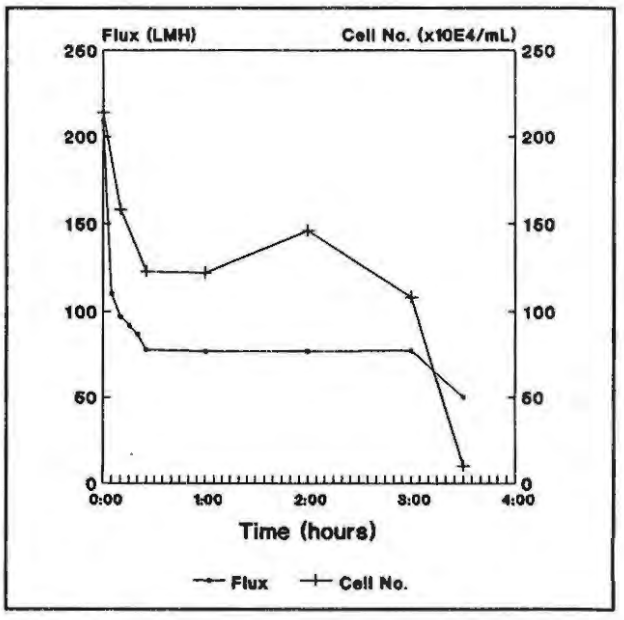


Figure 9.2 CFMF (diatomaceous earth pre-coat) concentration of *D. salina* comparing changes in cell number and permeate flux rate over time (LMH=litres.m.$^{-2}$ h^{-1}).

Figure 9.3 CFMF concentration of a *D. salina* culture. The starting cell number was 40x10^{4} cells.mL^{-1}, a cell concentrate of 1.1 % solids dry weight (SDW) and permeate of 4 (NTU) nephelometric turbidity units was produced.

Filtration pressure rose during the run from 350-400kPa and the pressure differential across the filter (P-inlet:P-outlet) was 1.6:1. The process produced a clear permeate without the appearance of any cellular material, and the turbidity measurement of 4NTU (nephelometric turbidity units) was within the range of municipal drinking water standards (ie. 5NTU). However, when β-carotene rich cells were shattered during filtration, the pigment was found to appear in the permeate.

The filter showed a 93% removal of bacteria from the permeate, measured as plate count colony forming units (CFU). Membrane rejection of dissolved inorganic medium components such as sodium, chlorides and phosphates was relatively small. The concentration in the reject volume of COD, TKN, nitrate and ammonia was predictable and was due to the accumulation of algal solids.

Table 9.1 Analysis of *D. salina* culture, permeate and concentrate produce by diatomaceous earth pre-coat cross flow microfiltration.

	Culture	Concentrate	Permeate
pH	7.9	7.4	8.3
COD $mg.L^{-1}$	2048	3348	11496
TDS $mg.L^{-1}$	84540	81600	77670
TDIS $mg.L^{-1}$	75040	72510	70530
TKN $mg.L^{-1}$	56	132	29
Nitrate $mg.L^{-1}$	1.4	3.5	1.4
Ammonia $mg.L^{-1}$	2.6	6.0	0.5
Chloride $mg.L^{-1}$	46807	45389	44397
Sulphate $mg.L^{-1}$	4009	3347	3507
Sodium $mg.L^{-1}$	40000	38000	36000
Phosphate $mg.L^{-1}$	73	71	72

While the failure of this system to produce a viable cell concentrate was consistent through many attempts at optimization, the high quality of the permeate indicated a possible application for this process in clarifying water containing suspensions of algae (figure 9.3).

9.4.2. Cross-Flow Ultrafiltration (CFUF).

The same adaptations to the filter rig were used with the attachment of a 9mm polyethersulfone-coated as described above, with a membrane tubular ultrafiltration unit replacing the CFMF flexible hose filter.

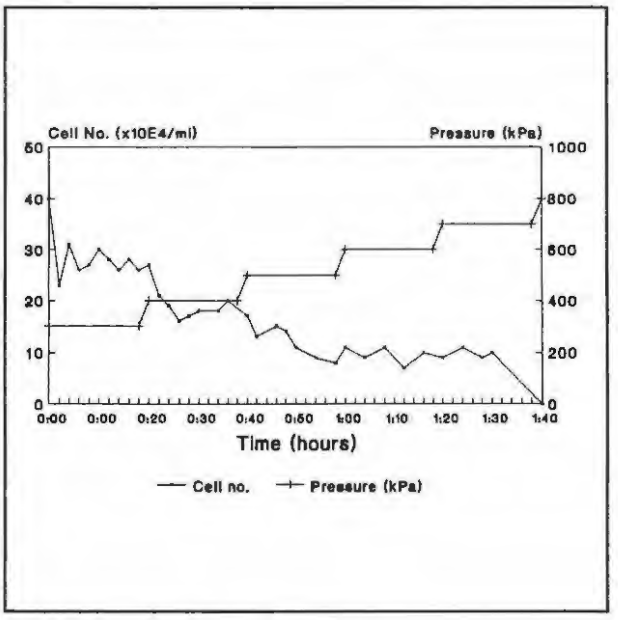


Figure 9.4 The optimization of operational pressure in CFUF separation of *Dunaliella* cells. The system was operated in permeate return mode and cell number was measured against a stepwise increase in operating pressure (range 300-800kPa).

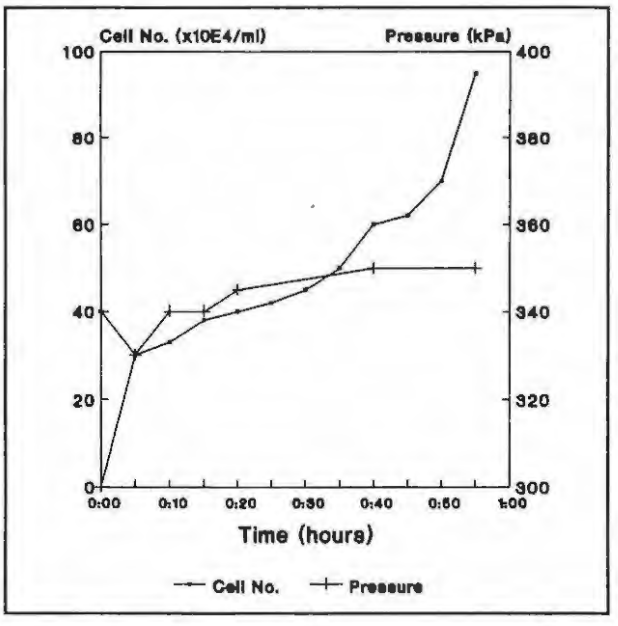


Figure 9.5 CFUF concentration of a *D. salina* culture where cell number was measured against an increasing operating pressure (range 300-400kPa).

Operational pressures were optimized (figure 9.4), using a culture of *D.salina* in ASW, by varying pump speed and assessed by measuring cell damage while the system was functioning in a non-concentrating, permeate return mode. A direct relationship existed between pressure and cell damage which was found to occur when

the system was operated at pressures above 400kPa. Where the CFUF system was operated in the cell concentration mode, an initial fall in cell numbers on starting filtration was observed, and is consistent with the establishment of a polarization layer on the inner surface of the filter coat (Tutunjian,1985). This was followed by a consistent rise in cell numbers. The results showed a pressure optimum producing least cell damage over the range 300-400kPa (figure 9.5). This optimum operating pressure was confirmed in all subsequent studies with an average cell loss usually falling between 0.5%-5% SDW.

The pressure differential across the filter was about 35:1 with outlet pressure 10-15kPa; flux average was 35L.minute^{-1} at a feed rate of approx 445L.hour^{-1}. A cell concentrate of 1.5% SDW was produced (figure 9.6). A clear permeate was produced (3.2NTU) with a small load rejection of inorganic salts (see table 9.2 and figure 9.7). A bacterial load reduction of 99.5% was measured as plate count CFUs.

Figure 9.6 Cell concentrate (1.5% SDW) and permeate produced by CFUF concentration of *D. salina*.

Table 9.2 Analysis of *D. salina* culture, concentrate and permeate produced in the cross-flow ultrafiltration separation of cells.

	Culture	Concentrate	Permeate
pH	8.2	8.0	8.1
COD $mg.L^{-1}$	2457	4141	1568
TDIS $mg.L^{-1}$	7750	7833	7741
Salinity 0/00	72	72	72

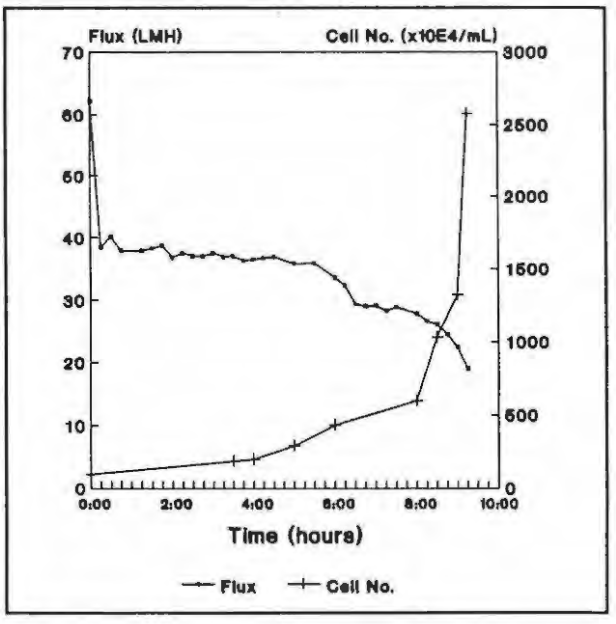


Figure 9.7 CFUF concentration of *D. salina* cells comparing cell number and flux rate.

Cells could be seen, by microscopic examination, to become rapidly immotile and rounded after the initiation of filtration. This condition was reversible, with the full cell number returning to a motile condition within 2-12 hours.

The effect of the CFUF cell concentration process on *Dunaliella* viability was assessed by comparing the growth rates and cell yields of cultures before and after the treatment. Cells were diluted back to starting cell numbers in fresh growth medium and figure 9.8 shows a growth curve of treated cells performing similarly to untreated controls.

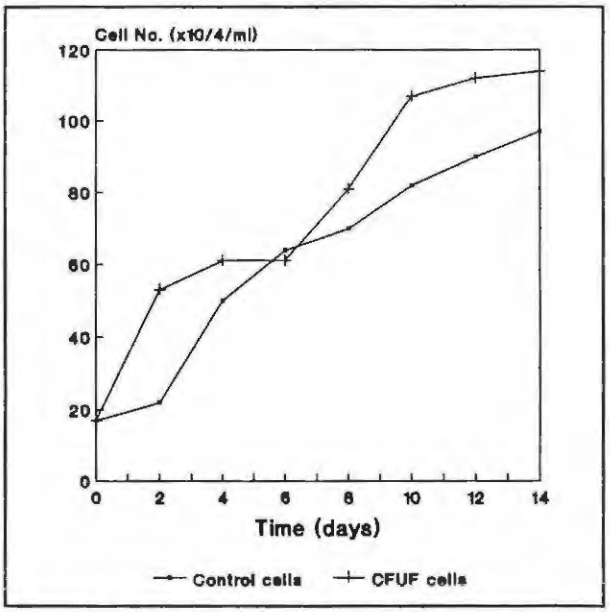


Figure 9.8 Growth of a CFUF-concentrated *D. salina* culture, diluted in fresh ASW medium to starting cell number, and compared to an untreated control.

The effects of CFUF on the subsequent β-carotene producing ability of by *Dunaliella* cells was evaluated by suspending the CFUF-concentrated cells and the control cells (removed from M11 growth medium by centrifugation at 3000g for 10 minutes) in stress medium (nitrogen-deficient, 3.5M NaCl) and incubating under standard conditions (figure 9.9). The CFUF treated cells again performed slightly better in terms of β-carotene produced per cell, possibly indicating stress effects of the process. The change in the β-carotene:chlorophyll ratio in CFUF-concentrated cells resuspended in stress medium is illustrated in figure 9.10.

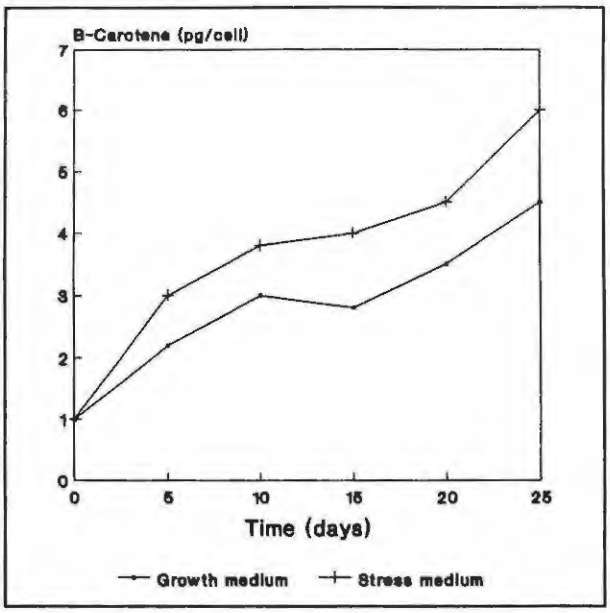


Figure 9.9 Comparison of β-carotene production in a CFUF-concentrated *D.salina* culture. Cells were diluted to starting number in nitrogen-deficient stress medium, and compared to untreated cells similarly resuspended in stress medium.

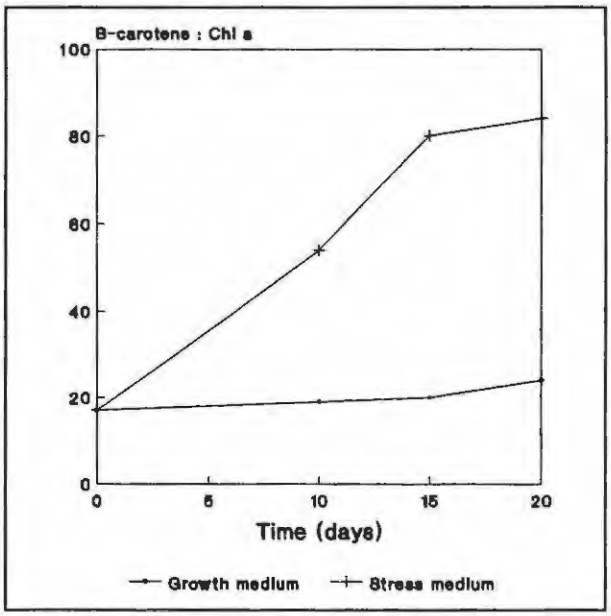


Figure 9.10 β-carotene:chlorophyll ratio development in a CFUF-concentrated *D. salina* culture. Cells were diluted to starting number in nitrogen-deficient stress and ASW growth media.

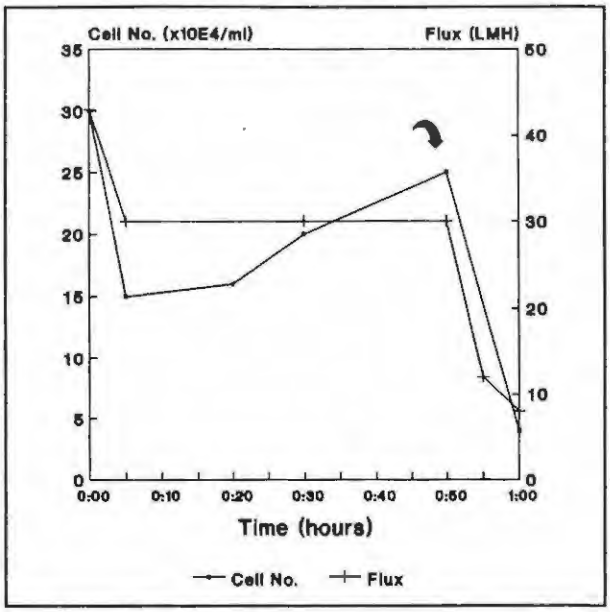
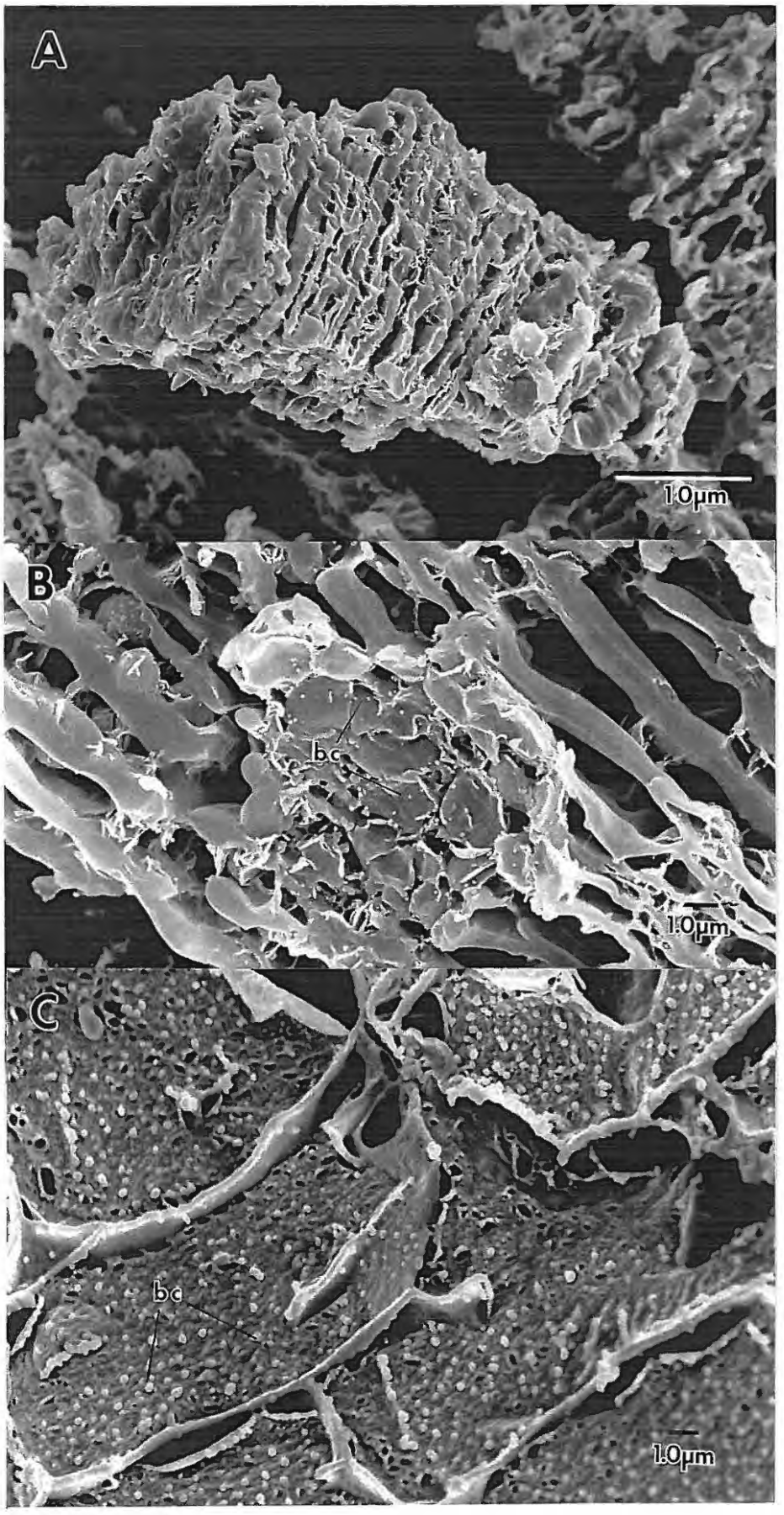


Figure 9.11 CFUF concentration of *D. salina* cells. Cell shattering was initiated at t=50 minutes by opening valve V2 (figure 9.1).

Figure 9.11 shows a CFUF cell concentration run with increased cell number plotted against flux rate. An initial rapid fall in flux rate is followed by a steady state and then a gradual decline over a period of hours. The demonstration that the flux decline was due to membrane polarization is shown in figure 9.11 where the concentration function was terminated after valve V2 (figure 9.1) was partially opened to produce the cell shattering effect already noted. Both cell number and flux rate fell rapidly. This result, showing cell shattering across the pressure regulation valve, may explain why previous attempts at the optimization of this system were unsuccessful. The pressure differential across the system of 35:1 is in sharp contrast to the 1.6:1 for the CFMF where cell loss was substantial. While the cells appear to be able to withstand sudden pressurization on pumping, the reduction of pressure should take place gradually across the full length of the system.

The β-carotene from cells shattered by partial opening of valve V2 is rejected by the polyethersulfone filter and does not pass through with the permeate which was shown by spectrophotometric measurement at 450nm. Ben-Amotz *et al.* (1982) have shown that β-carotene globules (diameter 150nm) occur in the interthylakoid spaces of *D.salina* and are released in a globular form on osmotic shock or rupture of the cells. An examination of SEM micrographs of cell debris (figure 9.12a,b), although not entirely convincing, tended to confirm the prior observation and showed a relative absence of carotene globules where fragments were compared with freeze fractured controls (figure 9.12c). It was assumed that if β-carotene does not remain bound to cell fragments after shattering, it is possible that the lipid globules are rejected by the hydrophillic polyethersulfone membrane.

Figure 9.12 (a)&(b) SEM micrographs of the freeze fractured and etched cell debris of a carotenogenic culture of *D. salina*. Cells were shattered during the operation of CFUF with pressure release across valve V2 (figure 9.1). β-carotene droplets (bc) are sparse but present. **(c)** SEM micrograph of the control freeze fractured carotenogenic *D.salina* cells showing the presence of β-carotene droplets (bc). Cellular level was 30pg.cell^{-1}.


A
1.0µm
B
bc
1.0µm
C
bc
1.0µm

(A)

(B)

Figure 9.13 (a&b)Technical scale pilot plant ultrafiltration rig (Membratek Pty Ltd Paarl) located on evaporation ponds at WTC, Wellington.

9.4.3. Scale-Up Evaluation of Cross-Flow Filtration.

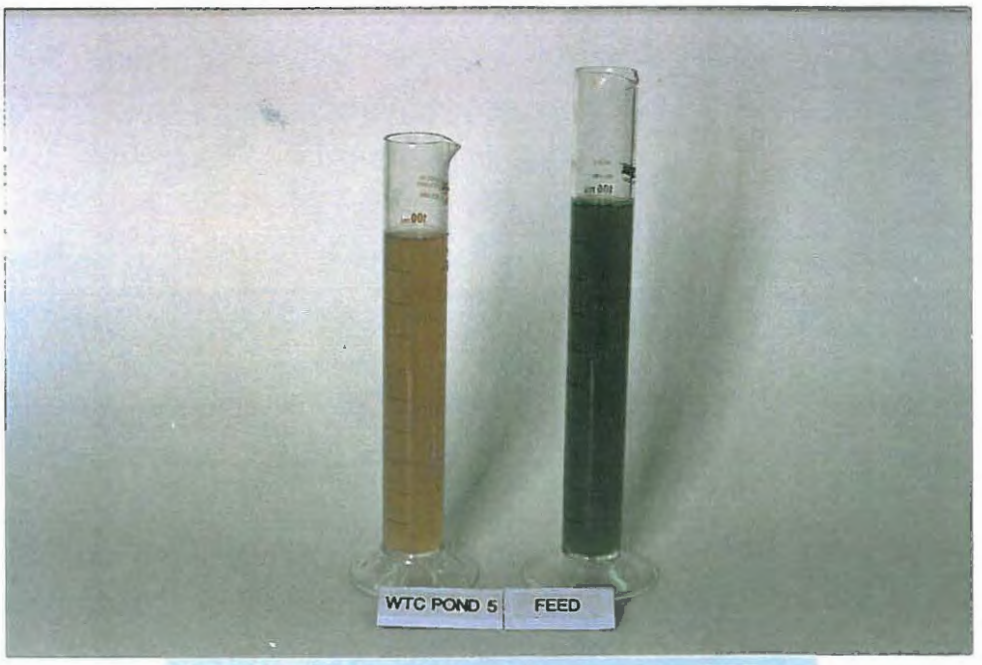
The laboratory studies reported above were performed using *D.salina* cultures in defined media, grown in 20L glass flasks and $1m^2$ paddle ponds. Further scale-up performance studies were undertaken at a tannery evaporation ponding installation to evaluate the potential of these systems as practical, industrially scaled cell separation techniques. Aspects of interest included not only the recovery of algal products, but also the ability of the systems to handle tannery evaporation pond liquors and the clarification effects where partially turbid effluents and pond liquors are to be used as a growth medium for the selective production of halophilic algae.

9.4.3.1. Cross-Flow Ultrafiltration.

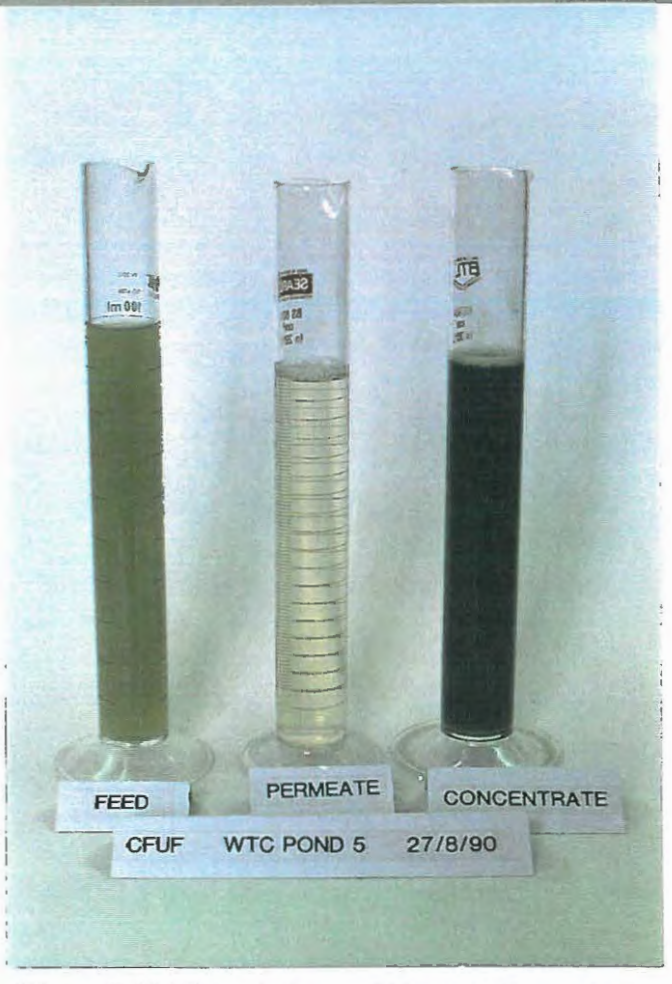
The cross-flow ultrafiltration rig used in this scale-up study was an industrial scale-up unit loaned by Membratek Pty. Ltd., Paarl (figure 9.13a,b). The filtration unit consisted of six $1.5m^2$ polysulfone-coated tubular ultrafiltration modules arranged in parallel, with tube diameter 9mm and tank capacity 660L.

Evaporation ponds numbers 5 and 8 (see figure 2.1) representing two stages of the evaporation cascade, with different constituents and supporting different algal populations, were used in this study. At the time of the trial the evaporation pond 5 contained a natural bloom of *Dunaliella* ($50x10^4$ cells.mL^{-1}) which was provisionally identified (by comparison with CCAP type strains) as a mixed culture of *D.salina* and *D.viridis* (figure 9.14a). The ponded liquor (table 9.3) contained a substantial component of dissolved organics which, together with the saline component, produced a slightly viscous consistency. It also supported a fairly heavy population of halophilic bacteria which gave the liquid a brigbt red colour when separated from the algae (figure 9.14a). Pond 8 contained a less dense algal population consisting primarily of *Spirulina* with the balance composed mainly of *Dunaliella* (also see figure 9.17). The liquor contained even higher levels of organics and salinity than did pond 5, with a very pronounced viscosity to the medium. The red colour was less intense.

For the first 2.5 hours of the CFUF run (figure 9.15), the system was set to operate at 10% recovery in the feed and bleed mode ie. single cycle with no accumulation of concentrated cells. The flux rate showed an uncharacteristic initial rise during this period with an average of about 50LMH ($L.m^{-2}.hour^{-1}$). The system was then switched to concentration cycle (arrow, figure 9.15) and run to 93% recovery, producing an algal slurry of 2.5% SDW (figure 9.14b). Cell viability studies were not undertaken, but microscopy showed a cell concentrate with limited cell damage. The flux rate fell gradually over the remaining period to 30LMH and the permeate analysis for both cycles remained the same. After the run the flux rate was fully restored on Biotex soap washing of the filter, indicating no permanent fouling of the membrane. These results suggest that flux reduction was concentration related, rather than due to a cell shattering effect.



(A)



(B)

Figure 9.14 (a) Samples from pond 5 WTC, Wellington, showing the presence at different levels of a *Dunaliella* bloom (right) and *Halobacterium* (left). **(b)**Samples of feed, permeate and concentrate from the CFUF scale-up evaluation study of algal separation on pond 5.

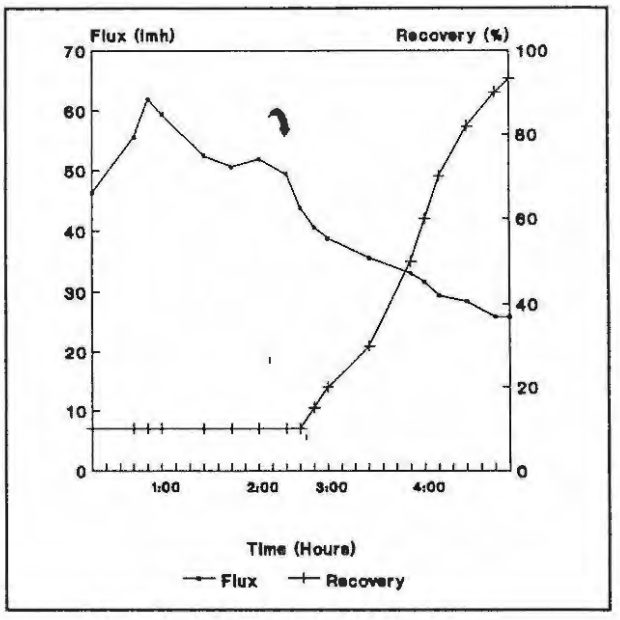


Figure 9.15 CFUF separation of *D. salina* growing in WTC evaporation pond 5. Arrow indicates commencement of the cell concentration cycle.

The system demonstrated an effective removal of suspended solids from the permeate (246 to 18mg.L^{-1}) and the concentration of algae (1615mg.L^{-1}) and of organics measured as COD, PV and total nitrogen (table 9.3). While chloride and conductivity measurements appear to show no rejection of salinity by the membrane, TDIS values tend to contradict this conclusion showing a slight reduction in the permeate. Dissolved solids, by contrast, show no membrane rejection in comparing feed and permeate.

Table 9.3 Analysis of feed, concentrate (at 93% recovery) and permeate from the scale-up evaluation of CFUF concentration of *D. salina* from pond 5, WTC. The permeate quality produced by CFMF treatment of the same medium is also reported.

	FEED	CFUF		CFMF
		93% REC.	PERM.	PERM.
pH	7.8	7.2	8.4	8.2
Conductivity $mS.m^{-1}$	2360	2380	2300	2400
PV $mg.L^{-1}$	145	768	76	79
COD $mg.L^{-1}$	480	3960	580	700
Nitrogen $mg.L^{-1}$	140	448	129	123
TDS $mg.L^{-1}$	1680	1930	1660	1750
TDIS $mg.L^{-1}$	620	530	420	1030
SS $mg.L^{-1}$	256	1615	18	11
Chloride %	0.73	0.71	0.73	0.73
Sulphate $mg.L^{-1}$	920	577	1013	927

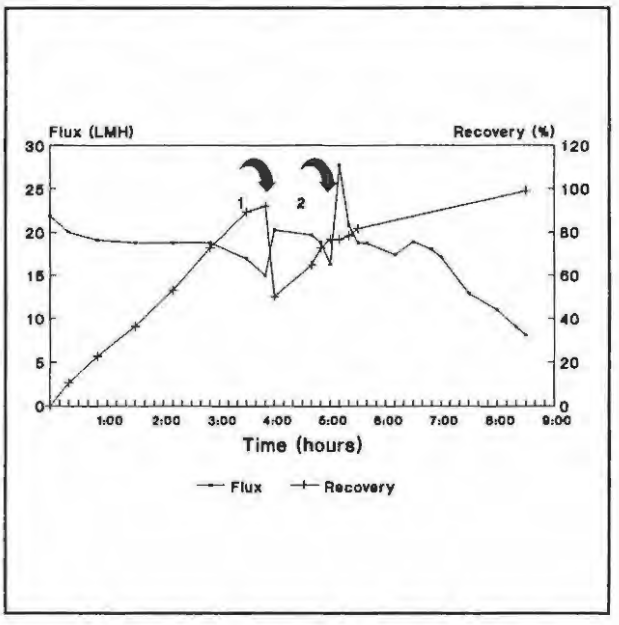


Figure 9.16 CFUF separation of a mixed culture of *Spirulina* and *Dunaliella* growing in pond 8. Arrow No.1 marks the addition of new feed to the concentrate, arrow No.2 marks a 2% Biotex wash, at 80 minutes, to monitor flux restoration.

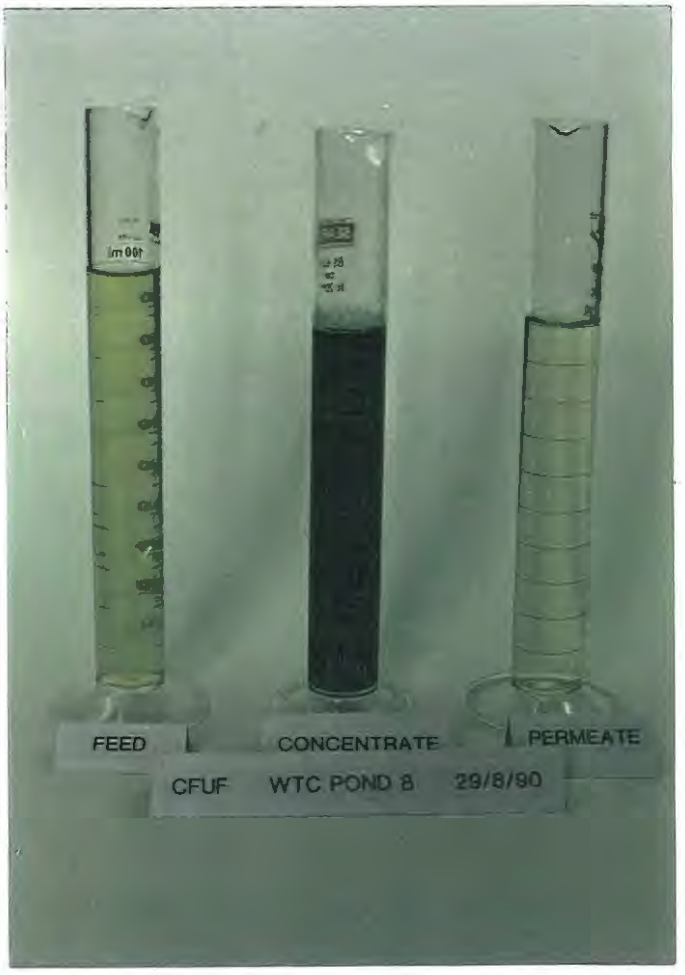


Figure 9.17 Samples of the feed, concentrate and permeate from the CFUF scale-up evaluation of algal separation on pond 8, WTC.

A similar scale-up evaluation of CFUF for the separation and concentration of algae was undertaken on pond 8 at WTC. Figure 9.16 reports the results of the system operated in the cell concentration mode, showing a gradually declining flux rate against rising solids recovery. The flux rate averaged about 18LMH which was considerably lower than the rate measured for pond 5. At 4 hours (arrow No.1) the cell concentrate was supplemented with fresh medium which produced a marked increase in flux rate. At 5 hours the process was interrupted and the membrane system washed with a 2% Biotex soap solution (arrow No.2). This again produced a significant improvement in flux rate demonstrating the reversibility of flux loss. The flux regeneration was short-lived and the rapidly resumed decline indicated a flux loss associated with concentrate build-up rather than irreversible fouling. At a solids concentration of between 1% and 3% (SDW), serious cell damage was noticed, possibly causing a dramatic fall in flux rate.

Suspended solids, chiefly algal solids, were concentrated up to 6.36% SDW. A good quality clarified permeate was produced with a limited rejection of both dissolved organic and inorganic solids, chlorides and conductivity (table 9.4 and figure 9.17).

Since lab studies on M11 medium had showed reasonably high flux rates at salinities of 1.5M NaCl for the CFUF system (35-60LMH), the reduced flux rates in pond 8 liquors is probably the result of the high organic load viscosity, rather than inorganic dissolved solids, which was quite low by comparison. The fairly substantial restoration of flux that followed filter washing also appears to indicate a concentration rather than cell shattering as the cause of the flux decline.

Table 9.4 Analysis of feed, concentrate (at 99.1% recovery) and permeate of the CFMF concentration of a mixed algal culture in pond 8 WTC. The quality of permeate produced by CFMF treatment of the same medium is also reported.

	FEED	CFUF		CFMF
		99.1% REC	PERM	PERM
pH	9.2	8.3	8.9	9.0
Conductivity $mS.m^{-1}$	4790	4530	4600	4540
PV $mg.L^{-1}$	67	1198	55	37
COD $mg.L^{-1}$	873	16848	686	686
Nitrogen $mg.L^{-1}$	25	1260	17	8
TDS $mg.L^{-1}$	4190	5390	3940	3890
TDIS $mg.L^{-1}$	3480	3240	3700	3320
SS $mg.L^{-1}$	21	1345	27	16
Chloride %	1.88	1.68	2.06	1.91
Sulphate $mg.L^{-1}$	1663	1712	1215	1215

9.4.3.2. Cross-Flow Microfiltration.

An evaluation of the CFMF system (diatomaceous earth pre-coat) as a technique for clarifying tannery evaporation pond effluents and for the removal of algal solids was undertaken at the same time as the CFUF scale-up study reported above. The configuration of the rig used was the same as for the laboratory evaluation described in 9.4.1. Both ponds 5 and 8 were used to draw liquid feed for the filter (tables 9.3,9.4). Rupture of algal cells was immediate as reported previously for CFMF, confirming the unsuitability of this technique for cell separation. A surprising rejection of dissolved solids was found with CFMF of pond 8 liquor. While a good quality clear effluent was produced, it was more turbid than that produced by CFUF. Flux rates of 60-80LMH were considerably better than those recorded for CFUF (figures 9.18,9.19). Microfiltration offers an effective technique for the removal of suspended solids from the evaporation pond liquors which would allow the recycling and use of the permeate in certain tannery applications, or as a medium for the culture of halophilic algae.

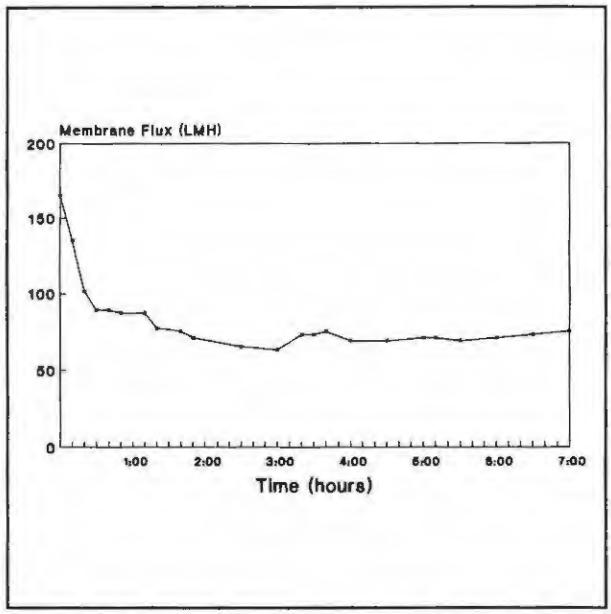


Figure 9.18 Membrane flux with CFMF clarification of pond 5 liquor, WTC.

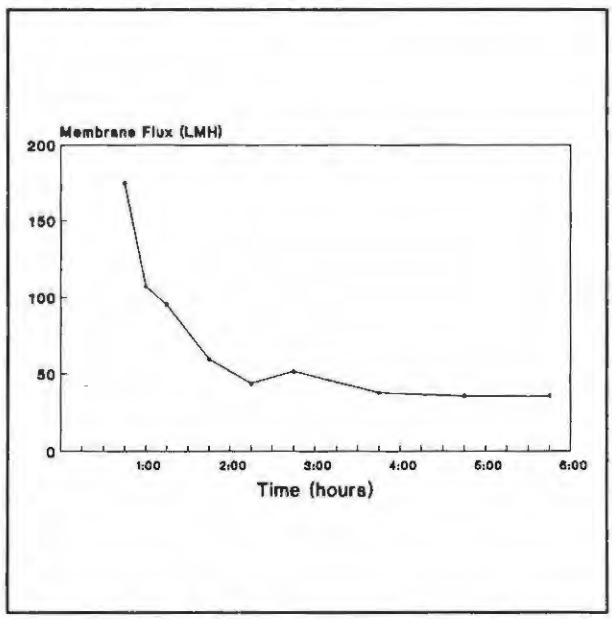


Figure 9.19 Membrane flux with CFMF clarification of pond 8 liquor, WTC.

9.4.4. CFUF - Diafiltration.

9.4.4.1. Curers Brine.

The CFUF diafiltration (cell washing) process involves the constant volume washing of filtration concentrates by the repeated addition of fresh water (or other medium) during the continuous operation of the CFUF concentration process. The scaled-up process was first evaluated with the Membratek rig, by the separation and washing of blood protein concentrates from Curers Brine Effluent, due to the unavailability of sufficiently

large *D.salina* culture volumes. The results are reported in figures 9.20, 9.21 and table 9.5. After the initial CFUF concentration of the effluent to 80% recovery, an addition of a further 50L raw feed was added to the remaining 15L concentrate (arrow 1, figure 9.20), resulting in a raised flux rate. When recovery had again reached 50%, a 50L volume of tap water was added, initiating the diafiltration process. Recovery was then run to 90% (arrow 2). At this point a further 20L tap water was added and recovery was again run to 90% (arrow 3). Note improvements in flux rate which accompanied each addition of wash water which again indicates that the flux decline is a concentration-related effect and not due to irreversible membrane fouling. Analytical results are reported in table 9.5 and the effectiveness of the process can be assessed by the 5.4 fold concentration of protein and the commensurate reduction of salt as measured by TDIS (4.97 times), chlorides (4.66 times) and conductivity (2.84 times).

9.4.4.2. Cell Washing.

The cell washing application of the CFUF - diafiltration process was evaluated using the WRC semi-technical scale pilot plant by the removal of salt from a 30L culture of *D.salina* ($40x10^4$ cells.mL^{-1}) where the salinity of the ASW medium had been elevated to 3M NaCl. Fresh ASW medium (1.5M NaCl) was added continuously to the feed tank T1 (figure 9.1) and 5.6 replacement volumes were required to reduce the salinity of the culture to 1.5M NaCl. Algal cells remained intact, although not motile, throughout the process. Full motility was observed to be restored within 2 hours after the procedure indicating no permanent damage. The total cell count had been reduced by 1.3% after that part of the polarized layer recovered by repeated washing had been taken into account.

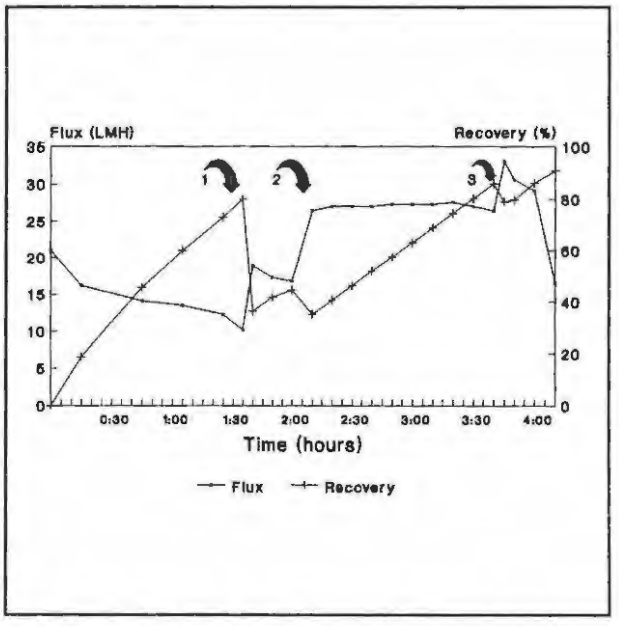


Figure 9.20 Diafiltration of Curers Brine. Solids were first concentrated and salts then removed by diafiltration washing with tap water. Arrow 1 = addition of supplementary feed; Arrow 2&3 = addition of diafiltration wash water.

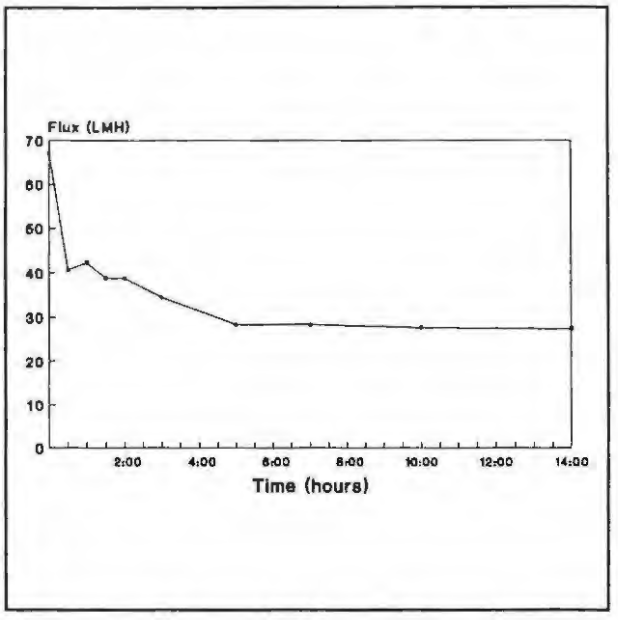


Figure 9.21 CFUF - Diafiltration of Curers Brine showing feed, concentrate and permeate. The feed was first concentrated to a 90% recovery and then washed with two volumes of tap water.

Table 9.5 Analysis of feed, permeate and concentrates (at 90% recovery and after diafiltration) in CFUF concentration and diafiltration washing of Curers Brine solids.

	FEED	90% REC	PERM	DIAFILT CONC.
pH	7.1	7.5	7.2	6.6
Conductivity $mS.m^{-1}$	15720	14880	15600	5530
PV $mg.L^{-1}$	2107	4985	1624	5478
COD $mg.L^{-1}$	10026	35984	7682	44032
Nitrogen $mg.L^{-1}$	1154	4365	543	5940
TDS $mg.L^{-1}$	27630	30740	25640	10600
TDIS $mg.L^{-1}$	24420	25330	24090	4510
SS $mg.L^{-1}$	226	1071	31	8614
Chloride %	14.36	15.25	14.93	3.27
Sulphate $mg.L^{-1}$	2352	2446	2663	2286

9.6. DISCUSSION.

The potential apparent in the multistage processing concept could remain as little more than an academic curiosity if the problem of adequate cell separation technology was not to be simultaneously addressed. The effective separation of *D.salina* cultures from high nitrogen levels present in proteinaceous effluents, is required before β-carotene synthesis can be successfully induced. The potential of the multistage approach is clearly not only limited to effluent-based systems but its implementation, together with adequate separation technology, could result in a substantial productivity improvement for the defined medium cultivation process. While CFMF was not found to be a successful technique for the harvesting or concentration of *Dunaliella* cells in a viable form, it was found to clarify a range of industrial saline effluents, with relatively high flux rates, and offers a practical method for the preparation of effluent-based growth media for the cultivation of *D.salina*

A possible cause of the shattering of algal cells noted with the CFMF system is the low differential between inlet and outlet pressures (1.6:1). By contrast the pressure fall across the CFUF system is much more favourable (35:1) with low outlet pressures of 10-15kPa compared to approximately 200kPa for the microfiltration system. Both pump speed and length of filter can be used to adjust pressure over a wide range for the fixed membrane systems such as the polyethersulfone tubular filters, thus minimising damage to algal cells. The microfiltration system requires reasonably high outlet pressures for the continuous maintenance of the diatomaceous earth dynamic membrane pre-coating. This inevitably produces a sharp pressure drop at the outlet point, which in turn results in the continuous fracturing of the fragile *D.salina* cells. Abrasive characteristics of the diatomaceous earth may also play a role.

It was found, as a general rule with the mechanical handling of *Dunaliella*, that while sudden and fairly large increases in pressure, are tolerated up to 600kPa, the sudden reduction of pressure across a short distance causes substantial cell damage.

After the preliminary optimization of the filtration rig the CFUF system was shown to operate effectively. Cell losses over the various CFUF trials undertaken fell into the 0.5-5% range, and in addition to actual cell damage, are probably associated with a fraction of the polarized layer not fully recovered when the system is shut down. Since this layer is related to filter area and not to volume through-put, the loss factor should decline with larger scale runs and this was generally found to be the case. These losses would be reduced and possibly eliminated with a more thorough post-process cleaning cycle, such as the use of sponge balls. A more accurate determination of cell losses will be required in further scale-up studies.

Falls in flux rate (with CFUF) were not permanent and rates were fully restored in each case with clean water washing. The flux falls observed in this study are consistent with the two stage explanation of membrane flux failure reviewed by Tutunjian (1985). It is thought that a thin polarized layer of particles (in this case algal cells) is established on the inside surface of the filter within the first minutes of initiating cross-flow filtration,

producing an initial rapid fall in the flux rate. This polarized layer forms an intimate part of the membrane filtration effect. The flux rate then levels off with the establishment of steady state conditions which persist until a second, more gradual decline sets in. This is due to the accumulation, by selection, of fine particles and debris of a size that results in the blockage of the interstitial spaces of the particles forming the initial polarized layer and the polyethersulfone coating itself. This effect can be induced prematurely by fracturing the algal cells across valve V2.

The filter blockage effects, reported by Ben-Amotz and Avron (1989a) to occur soon after the initiation of the filtration process, was not observed in this study after the system function had been fully optimised. It is possible that initial cell fracture in their system was responsible for the flux failure observed and led to their rejection of this process.

The successful concentration and recovery of viable cells with the CFUF system and its adherence to the performance predicted by other cell treatment applications, suggests that this process offers a useful cell harvesting technique for further evaluation in the large scale cultivation of *D.salina*. The results from diafiltration or cell washing of *D.salina* cultures and the Curers Brine also conforms to previously reported applications of the technique. Rosenberry *et al.* (1981) used CFUF for the removal of protein from erythrocytes, demonstrating the ability of the system to cope with extremely fragile cells and an approximately 99.3% medium change was effected in 5 volumes of filtrate. The processing of blood protein has been reported by Schmitthausler (1977). The cell washing technique offers a method of well demonstrated practical application for the transfer of *D.salina* cells from a high nitrogen growth medium to a nitrogen-deficient stress medium, that is required for the manipulation of metabolite induction in the multistage processing concept. The technique would allow the degree to which cells must be concentrated prior to the commencement of cell washing to be selected over a wide range. In other words, a theoretical point in the cell concentration process would be reached above which higher solids levels, causing falling flux rates, would be less economic than a larger wash volume which can be handled more efficiently at lower flux rates. The optimum relationship would need to be determined in larger scale-up studies.

Diafiltration will also enable the removal of salt by washing of final cell concentrates. This can be of importance where algal biomass is to be used as an animal feed, and where a reduction of equipment corrosion and abrasion caused by high salinity will contribute to lowered maintenance and replacement costs of the downstream product extraction plant.

Stabilized CFUF flux rates for *D.salina* cultures over the range 30-40LMH compares well with rates considered acceptable in industrial process, which may range between 5-50LMH (Kalk&Langlykke,1986). CFUF permeate flux rate for tap water is approx 125LMH (Membratek data sheet).

Various applications of the diafiltration cell washing technique have been reviewed by Gabler (1985), who identified a number of advantages of the process including continuous operation, time and cost saving compared to conventional processes such as batch centrifugation. The use of the same equipment to perform a variety of processing functions can have a significant cost reduction effect.

This versatility factor has been incorporated into the process design outlined in the following chapter.

An additional advantage of the use of CFUF for cell separation is the sterilization of the medium effected by the 80 000 MW cut-off maximum of the polyethersulfone membrane. This was confirmed by the substantial reduction of bacterial counts in the permeate and reflects the removal of larger particles such as algal contaminants, protozoal predators, fungal spores and dirt particles which can be responsible for a major limitation of the productive potential of algal cultivation systems (Richmond,1987). An effective mechanism for the control of contamination in *D.salina* cultures, such as that offered by CFUF permeate recycle, that does not require the elevation of salinity above algal growth optima, may be used to produce cell yields closer to the theoretical potential of the organism. The facility to select salinity growth optima has also been incorporated into the process design discussed in Chapter 10.

The experimental evaluation of the CFUF process has demonstrated that *Dunaliella* cells can be successfully concentrated and washed and that neither cell number, cell viability nor β-carotene production potential is seriously affected by either the CFUF cell concentration or diafiltration processes. These results indicate that larger industrially-sized scale-up trials are warranted. They also provide a more informed basis for the design of a scaled-up multistage *D.salina* production facility.

CHAPTER 10

PROCESS DESIGN

Summary.

The results of the research programme have led to the proposal and elaboration of a multistage processing concept for *D.salina* production. The findings have been summarised in process designs which demonstrate a utility for saline effluent wastes in algal metabolite production, and organic waste treatment and disposal functions. Application of these findings to the defined medium *D.salina* production process have also been noted. Aspects of novelty have been claimed in provisional patent applications.

10.1 INTRODUCTION.

The ultimate aim of biotechnology is to take an idea from the laboratory to the production plant and finally to the market place. The concept of a technology life cycle operating in this process has been noted by Hacking (1986), but in the case of algal biotechnology, given its relatively early stage of development, and with only a few scaled-up commercial plants operating world-wide, there is no generally accepted, defined procedure to determine this process of development (Benemann *et al.*,1987). Chapman and Gellenbeck (1989) have, nevertheless, suggested that requirements may not be all that different from those commonly accepted for terrestrial agriculture:

1. A complete and thorough understanding of the biology and the ecological needs of the cultivated species is imperative;
2. Techniques needed to improve the efficiency of the process require careful attention to growth and production requirements;
3. There must be a market of sufficient size and consistency to support continued improvement and advancement.

This they conclude, requires a multidisciplinary approach with a fusion of skills and effort from basic and applied biology, engineering and economics.

Borowitzka&Borowitzka (1989) provide one of the few reports generalizing procedures for a scale-up sequence of algal biotechnology programmes, and record their experience with the case study of β-carotene production from *D.salina* in open ponds at Hutt Lagoon, Western Australia. These procedures, common to other areas of biotechnology (Hacking,1986;Trilli,1986) can be evaluated as several distinct phases. The first concerns what

they have called the initial technical study. This should include a detailed evaluation of the biology of the process, establishment of broad parameters for growth medium constituents, development of product analysis methods, an assessment of suitable harvesting techniques and an evaluation of product extraction processes. With the establishment of biological and technical feasibility, the scale-up phase is undertaken through a number of stages. The Borowitzkas' report the use of a ten fold incremental scale-up procedure (common in fermentation biotechnology, Trilli,1986), to be manageable in biological and engineering terms for algal project development. The process is then completed by a piloting stage on which final engineering design, costing analysis and a decision to proceed to commercial production will be based.

Trilli (1986) has drawn attention to the value of subsequent scale-down procedures to effect improvements to an already functional process. A sensitivity analysis of costs will indicate where improvements in the process can lead to significant production cost saving and increases in productivity. This will give rise to a second series of development phases, where optimization may require a return to a study of the fundamentals of the process.

The study reported here has attempted to address the issue of algal production in saline waste within the requirements set out for the prosecution of a technical study. Within the context of the current stage of *D.salina* production technology development, aspects of the study also comply with the second process of scale-down optimization described by Trilli (1986).

Where the results provide a provisional indication of feasibility, and prior to the commencement of scale-up procedures, the technical study should be consolidated into a process design that provides for the transfer of the biological requirements into process operation concepts. This interface between the biology and the engineering technology of the process will not only direct the content of the scale-up programme, but also the costing and financial modelling on which a final decision to proceed to a commercial plant will depend. These latter aspects fall outside the scope of this report.

The following discussion deals with a number of ways in which the results of this research programme could provide a basis of the design of a novel approach to *D.salina* cultivation. While the primary purpose of the study was to evaluate the utility of saline effluents for algal production, a number of findings have emerged which also offer innovative application in defined medium production. Based on the results acquired, a multistage process has been proposed and certain areas of novelty have been claimed for conventional *D.salina* production: the use of saline effluent as an alternative media source and a role for algal culture in the management of saline effluents. The details of these claims are contained in Provisional Patent Applications (Rose,1991; Rose&Cowan,1991 a,b). Those aspects that concern the development of the process design are reported in figures 10.1 and 10.2.

10.2 METABOLITE PRODUCTION.

In principle, the design proposals presented could be followed for the production of β-carotene and/or glycerol and protein. Although this study has concentrated primarily on β-carotene, it has been accepted that the simultaneous production of *D.salina* biomass constitutes a glycerol resource in itself, the recovery of which has already been dealt with in some detail by Ben-Amotz (1980) and Chen and Chi (1981).

The demonstration in this study of micro-algal harvesting by CFUF, the recovery of biomass in a viable form and its effective separation from the growth medium (by operating the process in the diafiltration/cell-washing mode), provided a provisional indication of the feasibility of an enabling technique on which the practicality of the multi-stage concept largely depends. This enables the separation of biomass production and metabolite accumulation into individual unit operations where each aspect of the process can be more fully optimised than is possible in the "averaging" approach currently employed. The return of a virtually sterile permeate to the algal growth stage offers the advantage of infection control and hence the possibility of enhanced cell production at lower regulating salinities. A separate unit operation for effecting metabolite production offers the prospect of refining control over the stress induction process in *D.salina*. Extending a fundamental understanding of the physiology of the metabolite induction process, and its hormonal regulation at the cellular level in this organism, could provide a probe enabling a degree of control precision not currently available in the mass culture manipulation of the micro-algae. Together these factors appear to provide an attractive theoretical potential of yield enhancement which warrants further evaluation.

A provisional demonstration of the feasibility of these developments in the initial technical study, led to the proposal of an algal production process that can be run through a number of optional stages for which the descriptive term "multistage processing" has been applied. Figure 10.1 describes the process where metabolite production rather than an effluent treatment function is the principle objective.

10.2.1 Inoculum Development and Biomass Production.

Inocula can be prepared under near axenic conditions in small open ponds with elevated salinity (Borowitzka&Borowitzka,1989), or in closed tubular photobioreactors where infection can be contained at lower salinities (Pirt *et al.*,1983). This inoculum would be used to seed a series of short residence time, batch-type growth ponds, where rapid biomass production at lower salinities, optimum nitrogen levels and in an otherwise virtually sterile medium would favour the dominance of reasonably pure algal cultures. The use of high levels of inoculum (at least 10%) should further ensure both rapid growth and the dominance of the desired culture. Either a defined chemical formulation with or without amino acid supplementation, or an appropriate saline organic effluent could be used to constitute the growth medium. Smaller batch ponds will also offer more effective control of culture growth conditions than is possible in some of the large ponds (4000m^3) currently in use (Dodd,1986). The concept of batch production based on large inocula and rapid growth rates as a means of algal culture control has been documented by AQUACOP (1983) and Liao *et al.* (1983).

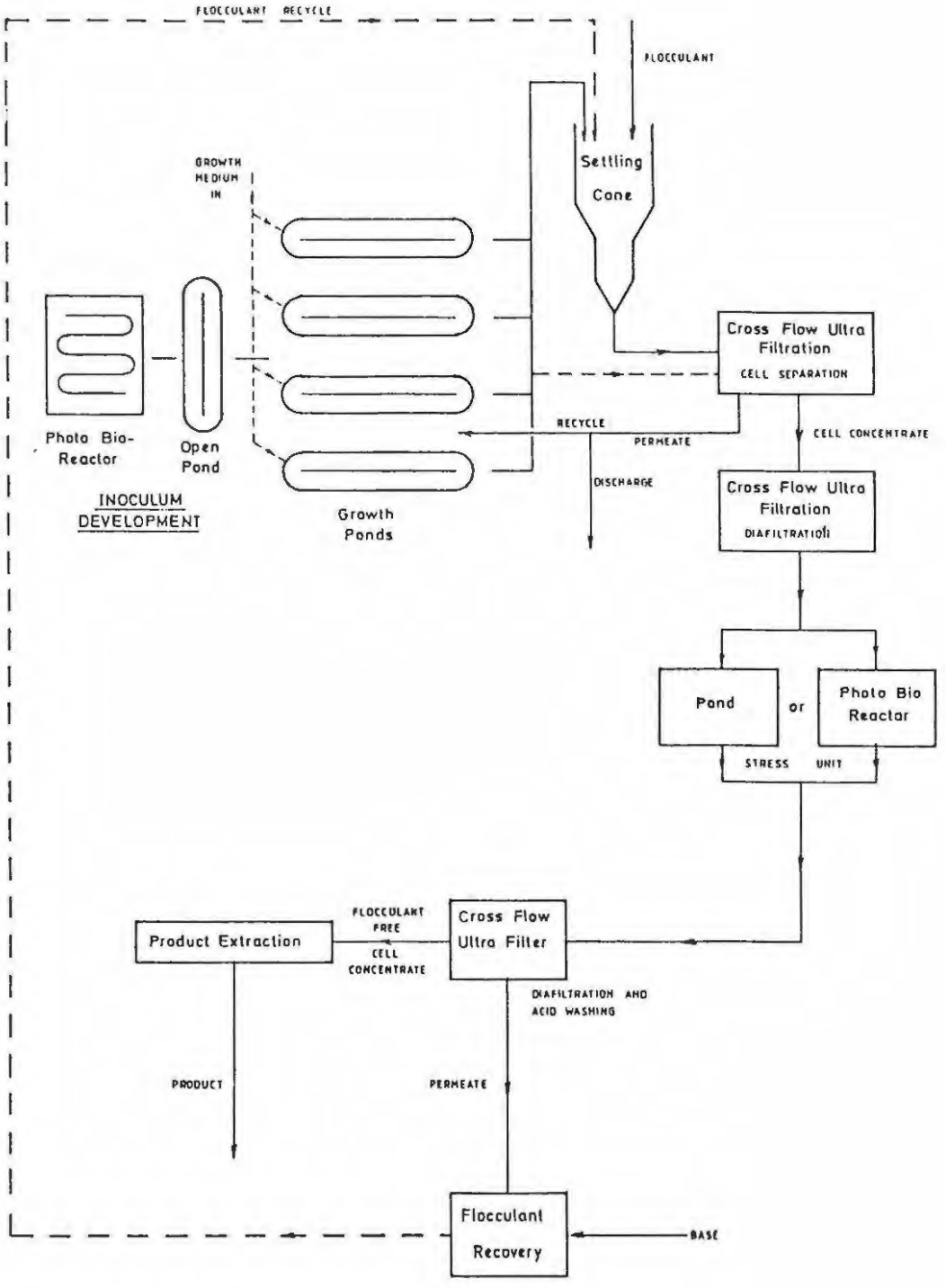


Figure 10.1 Flow diagram of the multistage process for cultivating *D.salina*. Cells may be cultivated to high densities under conditions of non-limiting nutrient supply, followed by transfer to a nutrient-deficient stress medium, with the induction of metabolite accumulation in the stress unit.

10.2.2 Cell Separation.

The well established advantages of flocculation can be optionally incorporated as a preliminary step in cell separation, depending on the quality of the final product desired. Where β-carotene is to be produced in effluent, no matter how controlled the source or the stringency of the extraction process, it is unlikely to be marketed as pharmaceutical grade. Where animal production is intended, the use of aluminium-based flocculants have been shown to be acceptable (Moraine *et al.*,1980).

Cross-flow ultrafiltration can be used, either with or without flocculation, to provide a cell concentrate of appropriate density whereafter, with the initiation of diafiltration, residual nitrogenous growth media can be removed and replaced with stress medium. The permeate can be returned to the growth unit in a near sterile condition and used on a multiple recycle basis.

10.2.3 The Stress Unit.

The cell concentrate may need to be diluted sufficiently to allow the resumption of photosynthesis and the production of β-carotene in an appropriate stress medium. Initial indications are that this should be deficient in nutritional nitrogen, and if hypersaline in addition, maximum conversion of starch to glycerol will also be effected.

The stress operation could conveniently be undertaken in either ponds or in tubular photobioreactors in which Pirt *et al.* (1983) have shown effective photosynthesis is possible at extremely high cell densities (around 20 $g.L^{-1}$). This would allow less re-dilution of cell concentrates and hence reduce the expense of the final recovery of biomass, preparatory to product extraction. Optimal cell densities to be maintained in the stress unit will require empirical determination.

An ability to formulate the stress medium, with the option of manipulating several stresses interacting synergistically, affords the possibility of a fine control of the metabolite induction process which has not been previously available.

The potential for developing the abscisic acid response (reported in chapter 8) as a physiological probe for monitoring the induction of an effective stress state in *D.salina* appears to hold promise for the further regulation of a separate stress unit. This could be especially important where additional stress factors may need to be manipulated in cells grown in an organic proteinaceous medium, and showing a substantial accumulation of nitrogenous reserves. In this case, stress induction and β-carotene accumulation could be separated by a period of time, during which the efficacy of the manipulation protocol would be more effectively monitored by a parameter other than product accumulation.

10.2.4 Product Recovery.

Biomass from the stress unit will require concentration to approximately 8% - 10% SDW prior to product recovery. This study has not dealt with the product extraction stage which is the subject of a separate report to be made from this laboratory. The CFUF/diafiltration process may again play a role here in washing concentrates preparatory to extraction. Provision has been made in figure 10.1 for the recovery and recycling of aluminium flocculant by a process of acid washing and precipitation with base addition, as described by Moraine *et al.* (1980).

10.3 PRODUCTION LINKED TO AN EFFLUENT TREATMENT FUNCTION.

The system outlined in figure 10.2 deals with an approach to saline algal aquaculture where the treatment of an organic waste is one of the primary objectives. The process would, in principle, function in a similar way to that described above.

The growth pond would function as an HROP effecting an organic load reduction of the appropriate effluent. The system could operate in a continuous mode with the careful regulation of salinity (provided from an evaporation ponding concentration process, integrated as part of the programme for the final disposal of salts). Alternatively, the addition of a back-up inoculum may be required. The algal product, together with the remaining refractory solids could be removed by flocculation and/or CFUF. At this point, depending on the nature of the effluent used and the product desired, if any, the cell concentrate could pass through diafiltration to a stress unit and thereafter to product extraction. This may involve β-carotene accumulation, but more effectively could also include glycerol and protein recovery. The remaining solids, together with algal waste, could be passed to an anaerobic digester where substantial mass reduction could be anticipated. The high carbon content of the algal component may improve the C:N ratios of the refractory solids remaining, and hence digestion rates. The algal/solids product of the HROP could alternatively pass directly to the anaerobic digester without a product recovery stage. Further refinement could include the use of the biogas produced to energise a furnace for the incineration of the final solids with the return of the combustion gas as a carbon source to the HROP.

10.4 SOLID ORGANIC WASTE DISPOSAL.

The potential versatility of the HROP system as a multi-purpose mass algal cultivation process has been previously noted, and a number of speculative applications of the concept have been advanced in this study. This potential could be further expanded with the utilization of saline effluents in a process for the disposal of problematic semi-solid organic wastes such as the secondary sewage sludges. In addition to a category of saline effluents already containing organics, such as those produced by the tanning industry, the disposal brines from desalination processes or saline effluent segregation (as proposed by the DWA "salts" project report) could also serve as HROP media. With the addition of organics to these media, similar organic load reduction effects to those already demonstrated for Hide Soak Liquor could be anticipated in this form of HROP.

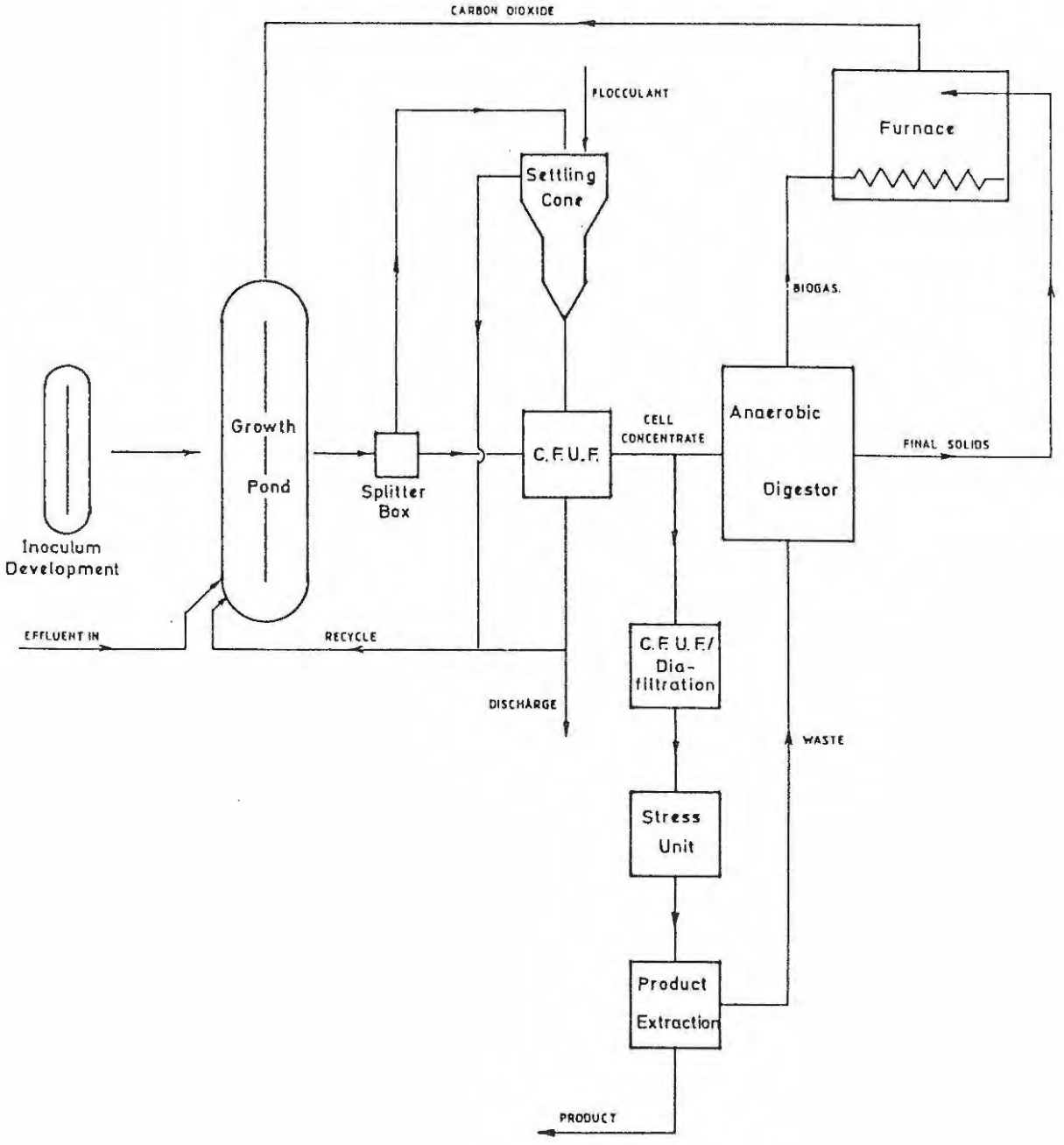


Figure 10.2 Flow diagram of the saline algal HROP process. Wastes that could be treated may either be organics present in effluent or organic solids added to saline waste water. Aspects of multistage processing may be added to facilitate the recovery of products and/or the final disposal of waste solids via an anaerobic digestion step.

This approach could constitute a novel function for saline waste-water impoundments, providing a co-disposal and a treatment site for both problematic organics and saline effluent wastes. Disposal of the final refractory solids component could be theoretically effected in the anaerobic digestion option, with the advantage of carbon supplementation from released glycerol and the algal biomass produced. A demonstration of the feasibility of such a system would provide a utility for saline waste water impoundments and a further incentive for dealing with the salinity problem at point source. While the concept is entirely speculative at this stage it is under further review in this laboratory and will be reported separately.

CHAPTER 11

CONCLUSION

Algal biotechnology, is at an early stage of development and, in common with most other areas of biotechnology, its benefits probably lie largely in the future. The subject is, therefore, necessarily speculative, research-oriented and dependent on a high degree of innovation. Hacking (1986) has noted that innovation is now widely recognised as being a cornerstone of the success of economic systems. However, the determinants of technological change and the problems of invention and innovation are poorly understood. Economic theory has tended rather to concentrate on the consequences of technological change.

In this regard, biotechnology research tends to operate in an area of uncertainty and has been characterized by "technology-push" rather than a "market-pull" approach. This has resulted in research that either does not justify the effort in terms of total market potential or competing technologies are available to do the job more efficiently (Hacking,1986). The type of work reported here needs to justify itself against this measure. However, effective research and development does not only rely on the generation of new inventions, but also on the timely recognition of losers and their rejection. In addition, if so structured, research programmes can also advance a fundamental understanding of biological processes which might result in the improvement of existing, economically justifiable projects.

Rosenberg (1982) has described models accounting for the development of technology which can be summarised within the concept of a "technology life cycle". This involves an initial enabling phase where fundamental work provides for future methods of production. A particular idea conceived within this framework then proceeds through the identifiable phases of development, application, and finally by the growth and maturation of the business. Scherer (1970), in dealing with the concept of innovation, has defined invention as an act of insight by which a new technical possibility is recognised and worked out in its essential and elementary form. The development which then follows involves the sequence of testing and modification required to prepare the original concept for commercial exploitation. Entrepreneurial and investment functions, involving the application of risk capital, will determine final viability.

It is within this frame of reference that the project reported here was undertaken as a study in feasibility, aimed at the modification and further optimization of an already established process. In terms of algal biotechnology project development concepts advanced by Borowitzka and Borowitzka (1989), it can be characterized as an initial technical study.

A fundamental research challenge that has faced algal biotechnology has been the problem of low productivity with the transfer of algal production from laboratory to large-scale outdoor conditions. This can, in turn, have a significant bearing on the marginal profitability of what are high production cost operations. This project, in the course of attempting to address the issue of a utility for saline wastes, has also demonstrated the provisional feasibility of process design changes which have a bearing on the productivity problem. In addition it has also been shown that the cost issue can be ameliorated, in part, with the use of effluent rather than defined media, and ponding facilities pre-costed to the waste treatment function.

The lack of reliable costing data available in the public domain, and hence the absence of a detailed costing analysis to be anticipated from a technical study of feasibility, is an evident weak point in this report. Hacking (1986) has considered the value of provisional estimates produced during the initial stages of biotechnology project development. He concludes that many potential projects can be screened by very approximate calculations based on rough estimates of production costs and the market value of products. This report does not proceed beyond that point.

The experimental aspects of the project focused on the application of algal biotechnology in the handling and treatment of saline effluents. The aim was to arrive at a provisional estimate of feasibility, to determine a value component for the approach and hence a utility for saline effluent wastes. A demonstration of utility could provide an incentive for the segregation of these wastes, with an impact on the wider problem of managing salinity in the public water system. In this regard tanning industry saline effluents served as the paradigm for the study. Among the appropriate halophilic algae, *D.salina* was selected, given the identification of its natural occurrence in this environment, the relatively well developed state-of-the-art biotechnology for its production, and a high value product providing a market justification for undertaking the programme.

It was demonstrated in this study that *Dunaliella* is able to grow, and grow well in certain saline tanning effluents, with an enhancement of cell production compared to defined media systems. This is accompanied by a substantial reduction in the organic load of the medium and has presented a different perspective on what has until recently been widely regarded as an obligate photoautotrophic organism. These findings also allowed a demonstration of the feasibility of a saline HROP based on *D.salina* which provided a waste treatment function similar to its fresh water equivalent. This, in turn, demonstrated a utility for saline wastes and hence provided a provisional verification of the research hypothesis - algal production is possible in saline organic effluents. Whether this could provide a further utility for waste brines depended largely on the recovery of a useful product.

It was shown that organic nitrogen present in the growth medium suppresses β-carotene accumulation by *D.salina*, and that the currently used process for averaging cell production and metabolite accumulation in a single production unit provided an inappropriate model for application to this particular situation. To address

this issue the presence of an actual uptake and utilization of organic nitrogen by *D.salina* needed to be demonstrated. The evidence acquired, while not unequivocal, provides a strong indication that *D.salina* does use organic nitrogen in the form of amino acids and protein, and mechanisms for its uptake and possibly also its release were demonstrated. Furthermore, a previously unsuspected form of positive control via glycerol release was identified. This may allow the organism to exercise a degree of external control over the heterotrophic breakdown of organic nitrogen in its medium, and hence also over its exposure to accumulating ammonia toxicity.

These observations could lead to incidental advances in defined medium *D.salina* production with the application of amino acid supplementation, and to the HROP process treating refractory organic wastes, given the release of substantial amounts of glycerol.

A re-evaluation of the multistage processing concept was undertaken to deal with the inhibition of β-carotene production in an organic growth medium. Application to the defined medium production process was also demonstrated. While the idea is not new, its previous evaluation was aimed at enhancing cell and metabolite yield through the manipulation of salinity stress. Both objectives prove to be insupportable. It was shown in this study that salt stress in the presence of high levels of nitrogen is particularly ineffective in inducing additional β-carotene accumulation.

The practical demonstration of CFUF, in both a laboratory and an industrial scale-up study, as an effective separation process for the micro-algae, and producing viable washed cell concentrates, provided an enabling technique for overcoming the conflicting production requirements for the *D.salina* system. The effective separation of cell growth and the metabolite accumulating stages of the process can allow the individual optimization of both biomass and metabolite production. In addition it was shown that a number of stresses acting synergistically can be applied under the conditions of low light that pertain in dense cultures.

The advance, at a fundamental level, of understanding stress signal transduction and the hormonal regulation of stress induced β-carotene production was identified as presenting a potentially powerful physiological probe for manipulating the stress process. This is of particular relevance where the accumulation of stored cellular nitrogen may be responsible for an unpredictable delay in the initiation of metabolite accumulation.

A provisional benefit estimate for the process has indicated a possible four-fold yield enhancement across the system. It has been argued that this advantage could improve the marginal profitability with which mass culture of *D.salina* has hitherto been associated.

The findings of the research programme were integrated into process designs that provide for both metabolite production and waste treatment functions. This demonstrates, theoretically at least, a possible utility for the integration of algal biotechnology into brine waste treatment and hence indicates a further verification of the research hypothesis.

The results of this programme, if successfully scaled up, could have a wider bearing on the development of algal biotechnology and address certain of the long term and possibly futuristic objectives identified for the field by Richmond (1986a) and Benemann (1989). Algal production, as it has currently developed, is a high cost operation and only a few speciality products have warranted large scale practical application. The potential cost credits associated with waste disposal have been identified as an important advantage of the HROP process which could have a decisive influence on the problem of algal production costs.

The saline HROP, provisionally demonstrated here, offers the additional advantage over its fresh water equivalent, that salinity can be manipulated to ensure the dominance of a single algal species, with the benefits of stable and predictable production. This is an essential prerequisite for the recovery of commercial viability of any algal production process. *D.salina*, however, offers a limited range of metabolites. Speculation on widening this range has been based on the potential of gene manipulation in the micro-algae. It can be suggested that having established a cheap, consistent and reliable crop cultivation approach to algal biomass production, based on *D.salina* culture in waste saline effluent, a following phase of development could involve the transfer of genetic potential to this organism, and the production of a range of metabolites beyond those currently recovered. Successful genetic manipulation based on a single organism could also, to some degree, change the current focus of algal biotechnology aimed at speciality chemical production. The optimization of the culture conditions and process requirements of a number of different organisms has been pursued for the production of a wider range of metabolites. The maintenance of many processes, with possibly widely differing biological and engineering requirements, is likely to be beyond the capability of any one particular production installation. This offers little versatility for responding easily to changing market requirements. Since the technology for developing the cultivation and recovery process for each particular organism currently presents the greatest obstacle to the development of algal biotechnology, standardization at the technical level may offer the most rapid source of advance. In this sense *D.salina* could fill the role of an algal genetic "workhorse" similar to that occupied by *Saccharomyces cerevisiae* in fermentation technology.

These broad prospects of saline algal culture have been noted by Richmond (1986a) and their linkage to a saline HROP proposed in this study could go some way to fulfilling Soeder's vision of the HROP providing a multipurpose system of algal production with a versatility comparable to land based agriculture.

Hacking (1986) has noted that biotechnology provides a "means of production" rather than being product-oriented in the way that computer or television technologies are. It is in this sense that the results reported here have attempted to evaluate the feasibility of broader enabling technology. While further work at this level is clearly required, and despite a rudimentary costing analysis, it appears reasonable to conclude that algal biotechnology, through providing a utility for brine wastes, could offer incentives and hence constructive advantages in dealing with the wider problems of salinity facing the public water system in this country.

POSTSCRIPT

The work reported in this study has been published in a number of reports as outlined in the Appendix. The ideas presented here are those of the author with the exception of the abscisic acid work which was evolved in collaboration with A.K.Cowan, who also assisted with aspects of the experimental work reported in Chapter 8. Aspects of the study reported in Chapter 3 were undertaken in collaboration with R.K.Laubscher.

A number of the concepts raised in this research programme have been identified as follow-up projects and are currently under investigation at the MSc and PhD level and are supported by grants from the Water Research Commission and Sasol Co. These include the following studies:

1. The saline HROP as a general purpose process for the disposal of solid organic wastes.
2. The disposal of final HROP solids by anaerobic digestion.
3. A determination of the glycerol production potential in the saline HROP treating organic wastes.
4. An evaluation of *Spirulina* production in tannery effluent.
5. The correlation of ABA with pigment changes related to metabolic events in the xanthophyll cycle and the further refinement of this system as a probe for manipulating the control of the stress unit.
6. Industrial scale-up and application of CFUF and aluminium flocculation in the operation of the *D.salina* multistage cultivation process.

APPENDIX

Publications

The following is a list of publications and reports that have arisen from the work presented in this study:

1. **Cowan AK, Rose PD** (1991) Abscisic acid metabolism in salt stressed cells of *D.salina*. Plant Physiol **97**:798-803.

2. **Cowan AK, Rose PD** (1991) Is there a role for abscisic acid in the response of the green alga *D.salina* var. *Bardawil* to salinity stress? Proc SA Soc Botanists, Pietermaritzburg.

3. **Cowan AK, Rose PD** (1991) Hormonal mediation of the response of *D.salina* to salinity stress. Proc Marine Estuarine and Fresh Water Ecosystems Conference, Grahamstown.

4. **Laubscher RK, Rose PD, Aken ME** (1990) Saline Tannery effluents as growth media for the halophilic alga, *D.salina*. Proc Sixth Congress SA Soc Microbiol, Stellenbosch.

5. **Maart BA, Rose PD, Laubscher RK** (1990) Tubular ultrafiltration - a process for the separation of fragile algal cells. Proc Sixth Congress SA Soc Microbiol, Stellenbosch.

6. **Rose PD** (1991) Treatment of saline effluent. RSA Patent Application No. 91/5069.

7. **Rose PD, Cowan AK** (1991) A growth and separation process for algae and to useful products obtained there from. RSA Patent Application No. 91/5070.

8. **Rose PD, Cowan AK** (1991) A process for the production of useful products from effluents. RSA Patent Application No. 91/5071.

9. **Rose PD, Tucker S, Laubscher RK** (1991) The role of glycerol release in Algal High Rate Oxidation Pond Treatment of saline organic effluent. Proc Marine Estuarine and Fresh Water Ecosystems Conference, Grahamstown.

10. **Rose PD, Maart BA, Tucker S** (1992) Cross-flow ultrafiltration used in High Rate Algal Oxidation Pond treatment of saline organic effluents with the recovery of products of value. Accepted for verbal delivery at IAWPRC Conference on Membrane Technology in Waste Water Management. Cape Town, March, 1992.

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