Physiology of acid stream algae

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PHYSIOLOGY OF ACID STREAM ALGAE

by

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To my

father, father-in-law,

pappa and mummy

DECLARATION

This thesis is entirely the result of my own work, except for the water chemistry data. It has not been accepted for any other degree and has not been submitted for any other degree.

(Signed) C Rajendran

C. RAJENDRAN

ABSTRACT

Sixteen axenic cultures of algae from acid streams of known water chemistry were produced without the use of antibiotics. These included four strains of *Euglena mutabilis* (Durham Culture Collection No. D464, D640, D641, D642). Strain D464, isolated from Brandon Acid Stream, Co. Durham, was selected for detailed study; the pH of the stream was 2.6.

The optimum yield of *E. mutabilis* D464 in the basal medium at pH 2.6, occurred at a photon flux density of 100 μmol m^{-1} s^{-1}. All four strains (D464, D640, D641, D642) had optimum yields between pH 3.4 and 4.0. Adaptation to low and high pH was checked with strain D464 and it was found that this strain could be adapted to grow at pH 1.5 and at pH 8.5.

The nutritional requirements and tolerance to heavy metals of strains D464, D640 and D641 was investigated under standard growth conditions and was found that Na (10 mg l^{-1} to 25 mg l^{-1}) improved yield. Strains D464, D640 and D641 grew in the vitamin-free basal medium, however in the strain chosen for detailed study (D464) adding vitamin B_1 and B_{12} improved yield.

Heterotrophic and photoheterotrophic growth was investigated using strains D464, D640 and D641 and was found that they utilize the following organic carbon substrates, glucose, fructose, sucrose, glycerol, lactic acid and acetate. Yield was least in acetate. These strains also utilized β-alanine, Dl-asparagine, glycyl-glycine, urea, uric acid and ethanolamine as sole nitrogen source in the presence of light but not in the dark. Using strain D464 phosphatase activity was checked and was found that this strain had both acid and alkaline cell surface phosphatase activity.

All three strains (D464, D640, D641) tested showed tolerance to varying concentrations of different heavy metals. Tolerance in the diminishing order appears to be as follows: Al>Mn>Pb>Ni>Co>Zn>Cu>Fe>Cd.

Factors influencing Zn toxicity were investigated using strain D464 and it was found that Zn toxicity was influenced by the size of the inoculum, the pH and the composition of growth medium. Na and Ca (above 10 mg l^{-1}) increased toxicity to Zn, while Cl (up to and including 75 mg l^{-1}) and glycyl-glycine reduced Zn toxicity. K (160 mg l^{-1}), Mg (200 mg l^{-1}) and Mn (80 mg l^{-1}) had no effect on Zn toxicity in strain D464.

ABBREVIATIONS

^{o}C	degree Celcius
l	litre
ml	millilitre
mg	milligramme
mol	molar
µm	micrometre
µg	microgramme
ng	nanogramme
h	hour
d	day
dw	dry weight
min	minute
$\bar{x}$	mean
sd	standard deviation
nm	nanometer
dia	diameter

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CONTENTS

LIST OF TABLES

Page No.

LIST OF FIGURES

Page No.

Page No.

LIST OF APPENDICES

CHAPTER ONE

INTRODUCTION

1.1 Acid Environments

1.11 General

Acid environments with their characteristic low pH, high acidity and low species diversity have been reported from Australia, Canada, Italy, Japan, South Africa, United Kingdom and United States of America (Lackey, 1938; Brock, 1969; Van Everdingen, 1970; Castenholz, 1973; Hargreaves *et al.*, 1975). Although most sites are from volcanic areas and therefore of natural occurrence, in recent years man's activities associated with the mining for coal, copper, lead, zinc, barite, manganese and gold, together with discharges from industries dealing with battery manufacture, tanning, chrome plating and the burning of fossil fuel in factories and electricity degenerating plants, have greatly increased the incidence of acid in fresh water bodies which hitherto have been alkaline or near neutral. Acidification of rivers due to coal mine discharge in Virginia and Tennessee in U.S.A., where several hundreds of miles of river water has become unsuited for drinking or recreational purposes, has been well documented (Barnes and Rombergen, 1968; Nicholas and Bullow, 1973). In England and Wales too, mining has led to an increase in acidity in some streams (Hargreaves, 1977).

1.2 Hydrogen Ion Concentration

Hydrogen ion is by far the most important factor in growth and reproduction as it affects the ionic state and therefore

the availability of nutrients to the organisms (Cholnoky, 1960). Cholnoky postulated that at low pH hydrogen ions may adsorb to particulate matter and displace cations in the exchange complex which may then leach out of the micro environment. The influence of pH on growth (Hargreaves and Whitton, 1976; Shehata, 1981) and the solubility of heavy metals (Van Everdingen, 1970; Hargreaves *et al.*, 1975) has been well documented. The concentration of hydrogen ion influence the solubility and therefore the availability of certain metals to the algae in the growth medium. Most metals in solution at low pH become precipitated at higher pH and thus become unavailable for the organisms. Harding and Whitton (1977) observed a reduction in Zn toxicity in *Stigeoclonium tenue* when the pH was raised from 6.1 to 7.6. In *Hormidium rivulare*, Say and Whitton (1977) found an increase in Zn toxicity when the pH was reduced from 8.0 to 3.0 and Shehata (1981) found a marked decrease in toxicity of Zn to *Anacystis nidulans*, when the pH was raised from 6.5 to 8.0.

1.21 Internal pH

The existence of a different internal pH independent to that of the external pH has long been recognised. Crude measurements of internal pH on crushed cells have been reported by using microelectrodes, indicators such as bromothymol blue and by the measurements of external and internal weak acids and bases (Caldwell, 1956; Waddel and Bates, 1969). Other methods used are the determination of the amount of fluorescence quenched in cells which have absorbed amines (Schuldiner *et al.*, 1972) and the use of 5.5 dimethyloazolidine 2-4-dione (DMO) a metabolically inert non-toxic weak acid that does not form complexes with proteins or lipids (Butler *et al.*, 1966; Cassin, 1974).

Using C^{14} DMO determination of the internal pH has been carried out in *Streptococcus lactis* (Kashket and Wilson, 1973), *Bacillus acidocaldarius* (Yamazaki *et al.*, 1973), *Thiobacillus ferrooxidans* (Amemiya and Umberit, 1974), *Chlamydomonas acidophila* (Cassin, 1974), *Chara corallina* (Walker and Smith, 1975), *Cyanidium caldarium* (Enami and Fukuda, 1975), *Chlorella pyrenoidosa, Scenedesmus quadricauda* and *Euglena mutabilis* (Lane and Burris, 1981).

It is very unlikely that the internal pH corresponds to the external pH in any organism. Thomas *et al* (1976), using fluorecine diacetate, reported that *Bacillus acidocaldarius* had an internal pH range of 2.0 to 7.0. In *Chara corallina*, Walker and Smith (1975) recorded a cytoplasmic pH of 7.7 when the external pH was 5.0 to 6.0, while in *Cyanidium caldarium*, the authors found a steady internal pH of 7.5 over an external pH range of 6.0 to 8.0. In *Euglena mutabilis* (Lane and Burris, 1981) found a wide range of internal pH ranging from 5.0 at very low external pH to 8.0 at higher external pH. An interesting feature in *Euglena mutabilis* was that it was able to regulate its internal pH up to external pH 6.0 and not beyond. Whereas the acid non tolerant *Scenedesmus quadricauda* and *Chlorella pyrenoidosa*, continued to maintain the internal pH levels near neutrality.

1.3 Nutrient Status of Acid Water

The nutrient status of acid mine water differs from site to site and in general is probably eutrophic (Hargreaves, 1977). Bennett (1969) reporting on the nutrient status of acid mine

discharge gave concentrations of 6.64 - 0.1 mg l^{-1} NO_3-N and 4.8 - 0.5 mg l^{-1} PO_4-P. Hargreaves (1977) in a survey of Brandon Acid Stream (Section 2.311) made over a period of three years found 3.2 - 0.044 mg l^{-1} NO_3-N and 0.63 - 0.01 mg l^{-1} PO_4-P. Carbon forms another important growth requirement in acid environments as solubility of CO_2 is reduced at low pH values. By adding CO_2 up to a concentration of 5.4 µg l^{-1}, Ohle (1981) found that the absolute photosynthetic maximum of 330 µg C l^{-1} was reached. But when the CO_2 supply was stopped, the rate of photosynthesis strongly decreased. Coleman and Colman (1981) too, found CO_2 to be a growth limiting factor in *Coccochloris peniocystis*. Beardall (1981) concluded that CO_2 in acid waters must be the only carbon species to cross the plasmalemma; thus suggesting that CO_2 to be a growth limiting factor for algal growth in acid environments, as CO_2 gets rapidly lost from acid water as it equilibrates with the atmosphere. Wilcox and De Costa (1982) suggested that CO_2 in acid lakes to be from the atmosphere and not necessarily from metabolic activities of other microorganisms. Although CO_2 seems to be limiting in acid environments Satake and Saijo (1974) and Ohle (1981) found the CO_2 content of acid lake water to be more than the theoretical value for CO_2 dissolved in acid waters.

In addition to macroelements that may be limiting in acid waters, Hunter (1972) observed that trace element requirement by many microorganisms increase at low pH.

1.4 Heavy Metals in Acid Water

A number of potentially toxic heavy metals including Zn, Cu, Mn, Fe, Al, Pb, Cd, Ni and Co in varying concentrations have been reported from acid waters worldwide (Van Everdingen, 1970; Hawley, 1971; Hargreaves *et al.*, 1975; Jansson, 1981). Van Everdingen reported a number of heavy metals in over 11 sites with pH 2.2 to 5.5 in the Kootenay National Park in British Columbia. Sheath *et al.*,(1981) found increased heavy metal concentration due to long term acidification. Stokes *et al.*,(1981) found increased mercury in fish in acidic lakes. Rasmussen and Sand-Jensen (1979) reported that iron occurs in high concentration with other elements in the order Mn>Zn>Ni>Pb>Cr>Cd. In addition to Fe, Hargreaves (1977) found high concentration of Al in Brandon Acid Stream (Table 2.6).

1.5 Sources of Acidity

1.51 Air borne

The burning of coal in industries and the resultant sulphur dioxide emission has greater implication in the acidification of water bodies as air borne acid particles are carried many hundreds of miles from the source, depending on the meteorological conditions prevailing at the time of emission. Many lakes in Sweden have become acidic in the past two decades, resulting in total fish kill in some (Brosset, 1973; Henriksen and Wright, 1977). Long term fumigation by sulphur dioxide and sulphuric acid aerosols from nearby lignite burns has resulted in the acidification of some tundra ponds at the Smoking Hills in N.W.T. in Canada (Sheath *et al.*, 1981).

1.52 Water borne

The oxidation of inorganic and organic sulphur is believed to be the cause of acid production. The acid produced is predominantly sulphuric and hence is thought to be due to the oxidation of any sulphide from either mineral or organic source. Of the sulphides, mascarite and pyrite are most important. Pyrite differ from mascarite in that the latter alters much more readily to malanterite ($FeSO_4\ 7H_2O$) and limonite ($Fe_2O_3\ ^nH_2SO_4$) on oxidation (Riley, 1960). Sulphuritic materials in combination with iron, may in theory, oxidise to form sulphur dioxide or sulphuric acid depending on the availability of water.

Thus: 1. $FeS_2 + O_2 \longrightarrow FeSO_4 + SO_2$

2. $FeS_2 + 2H_2O + 7O_2 \longrightarrow 2FeSO_4 + 2H_2SO_4$

This means that not only sulphuric acid but also ferrous sulphate will be produced. In acid streams ferrous sulphate in the presence of sulphuric acid and oxygen oxidise itself to ferric sulphate.

3. $4FeSO_4 + 2H_2SO_4 + O_2 \longrightarrow 2Fe(SO_4)_3 + 2H_2O$

When the acid concentration in the stream is reduced due to other effluents ferric sulphate will be hydrolysed thus:

$$Fe_2(SO_4)_3 + 6H_2O \longrightarrow 2Fe(OH)_3 + 3H_2SO_4$$

Ashmead (1955) observed that the oxidation of FeS_2 in sterile water was slow compared to unsterile mine water and leads to a hypothesis that the production of acid in mine water was not purely a chemical reaction. Lundgren *et al.*,(1972) reported that *Thiobacillus - Ferrobacillus* group of bacteria are directly responsible for much of the acid produced in mine waste water as a result of oxidation of ferrous ions with the production of sulphuric acid. This is further confirmed by Fjerdingstad and Nilssen (1982). From the available literature it appears that the process of acid formation seems to be a combination of geochemical and biochemical processes. It is probable that the initial chemical process under wet or moist condition where

$$FeS_2 + H_2O + 7\ O \longrightarrow FeSO_4 + H_2SO_4$$

followed by bacterial action with *Thiobacillus ferrooxidans*, where

$$FeSO_4 + O + H_2SO_4 \longrightarrow Fe_2(SO_4)_3 + H_2O$$

subsequent chemical action:

$$Fe(SO) + FeS \longrightarrow 3\ FeSO + 2\ S$$

$$2\ S + 6\ Fe_2(SO_4)_3 + 6\ H_2O \longrightarrow 12\ FeSO_4 + H_2SO_4$$

followed by bacterial action by *Thiobacillus* where

$$S + 3\ O + H_2O \longrightarrow 2\ H^+ + SO_4$$

1.6 Biological Components of Acid Waters

1.61 General

The absence of many of the common aquatic macrophytes is a characteristic feature of acid streams. Except for

Vallisneria sp., *Typha latifolia, Juncus oxycarpus, Eleocharis acicularis, E. obtusa, Sphagnum truncatum, Scirpus fluitans, Carex* sp., *Isoetes* sp., *Phragmites australis* (Heaton, 1951; Moor and Clarkson, 1967; Patrick *et al.*, 1974; Stokes *et al.*, 1981), a few bryophytes represented by *Drepanocladus fluitans, Dicranella* sp., *Cephalozia bicuspidata* and *Jungermannia* (Hargreaves, 1977; Wehr and Whitton, 1983), acid streams appear bare of vegetation. Yet acid waters contain a large diverse microbial population of yeast, fungi, bacteria and algae. Yeast represented by *Saccharomyces ellipsoides, S. guttalata, S. cerevisiae, Rhodotorula, Candida* sp., and *Trichosporum* (Starkey and Waksman, 1943; Cooke *et al* 1960). *Aspergillus, Penicillium, Trichoderma, Helminthosporium, Trichothecium* (Ehrlich, 1963; Weaver and Nash, 1968) form the major fungi in acid streams.

1.62 Bacteria

The presence of sulphur oxidising bacteria in acid streams was reported as early as 1919 by Powell and Parr. Waksman and Jaffe (1922), identified *Thiobacillus thiooxidans* and Temple and Koehler (1954) described and named an iron reducing bacterium *Thiobacillus ferrooxidans*. Leathen *et al.*, (1956) isolated *Ferrobacillus ferrooxidans* from bituminous coal mine waste water at pH 2.5 - 4.5. Uchino and Doi (1967) described *Bacillus coagulans*. Dugen *et al.*,(1970) isolated bacteria from acid mine waters of pH 2.0 - 4.0, which included *Bacillus, Micrococcus, Sarcenia, Crenothrix* and *Microsporium*. Other bacteria in acid waters are *Thermoplasma acidophilum*, whose cell membrance is in direct contact with the hot acid (Patrick *et al.*, 1970), *Bacillus acidocaldarius* (Doemel and Brock, 1971), *Thiobacillus organoparus*

which has a morphological similarity with *Thiobacillus thiooxidans* (Markosyan, 1973) and *Sulfobolus acidocaldarius* (Brock *et al.*, 1972).

1.63 Algae

As algae are primary producers in any ecosystem, their presence is vital for the establishment and continued maintenance of species diversity, especially in extreme situations created by the presence of acid in the environment. Surveys carried out by Lackey (1938), Steinback (1966), Weaver and Nash (1968), Bennett (1969) and Hargreaves *et al.*, (1975) provide a comprehensive list of algae found in acid streams. *Euglena mutabilis* is by far the most common species to be recorded in all acid situations, exception being its absence from Kootenay Paint Pots in British Columbia (Wehr and Whitton, 1983). Hargreaves *et al.*, (1975) recorded a 90% abundance of this species in a survey of 15 sites in England with a pH of 3.0 and below. Other species recorded include *Pinnularia acoricola* (71%), *Gloeochrysis turfosa* (61%). *Nitzschia subcapitellata, N. elliptica* var. *alexandrina, Eunotia exigua, Chlamydomonas applanata* var. *acidophila Hormidium rivulare* occurred in over 20% of all reaches surveyed. In extremely low pH below 2.0, *Chlamydomonas applanata* var. *acidophila* seem to dominate Fott and McCarthy (1964). *Chlorella, Stichococcus, Scenedesmus, Ulothrix zonata, U. subtilis* and *Navicula* sp. are also inhabitants of acid environments (Fott and McCarthy, 1964; Stokes *et al.*, 1973, Satake and Saijo, 1974). The eukaryotic algae, *Cyanidium caldarium*, a thermal acidic species has, too, been very well documented (Geitler, 1936, Doemel and Brock, 1971, Aldo *et al.*, 1981).

Blue green algae are conspicuous by their absence in acid streams, (Brock, 1973). A recent report by Lazarek (1980) referred to an algal mat consisting of *Lyngbya* sp., *Oscillatoria* sp., and *Pseudoanabaena* sp. in the sediments of Lake Gardsjor in S.W. Sweden; the pH of the lake water and not the lake sediment from which the algae were seen was found to be 4.3 - 4.7.

1.7 Photoheterotrophic and Heterotrophic Growth in Algae

Algae are considered to be primary producers because of their ability to photosynthesise, yet there are a number of them that lack the photosynthetic apparatus and hence depend on organic substrates for growth and reproduction. However, there are some photosynthetic algae that utilize organic substrates as energy source both in the light and also in the dark. Rhodophyceae and Phaeophyceae both have been cultivated and none show any ability to utilize organic carbon in the dark (Droop and McGill 1966). So are pelagic diatoms and coloured Dianophyceae (Provasoli and McLaughlin, 1963), Khoja and Whitton (1971) reported 17 heterotrophic Cyanophyceae out of a collection of 24. In the Chlorophyceae, Chloroccocales have been reported to be able to grow in the dark (Beijerinck 1890 referred to by Droop (1974). Euglenophyceae as a group has not been fully tested for heterotrophy though different strains of *Euglena* have been experimented with.

Algae may appear to be obligate phototrophs, yet it is very difficult to demonstrate this. Stewart (1974) reported Droop's reference to the Scottish Marine Biological Association's collection in which 91 were apparently obligate phototrophs. This may be due to some vital requirement such as

the right concentration of the substrate. *Prymnesium parvum* a member of the Haptophyceae which was thought to be an obligate phototroph has been now shown to utilize glycerol in the dark provided the substrate was over 0.25 M (Rahat and Jahn 1965; Rahat and Spira (1967). Anita *et al.*,(1969) found the same for a marine cryptomonad *Chroomonas salina*.

Acetates and sugars have been tested on different strains of *Euglena*. The Manix and Vischer strains of *Euglena gracilis* used only acetic acid and butyric acid, while Pringsheim's strain of the same species used a great range of straight-chain fatty acids (Cramer and Myers 1952). Provasoli (1938) found his strain *E. gracilis* var. *europhora* used lower branched acids and alcohols as well. Sugars and sugar alcohol are not used by any of the above strains. Further Cramer and Myers (1952) and Hurlbert and Rittenburg (1962) found *E. gracilis* var. *bacillaris* could utilize the carbon skeleton of some amino acids as sole carbon source while Pringsheim's *E. gracilis* var. *saccharophila* could utilize both acetate and glucose.

Although the necessity for carbon in most natural environments does not present a problem to most algae, yet in low pH environments where CO_2 and light are limiting factors the ability to utilize organic substrates will enhance its chance for growth and survival

1.8 Factors Influencing Heavy Metal Toxicity

1.81 Nutrients and Heavy Metals

The effect or the effects of a particular metal on an organism may or may not be seen, as often organisms are

able to alter their environment to suit themselves or transform themselves (mutate) to suit the environment. Either way the effect of the metal may remain obscure. This is further complicated by the presence of other metals which may be antagonistic, thus masking the effect of the metal. Abelson and Aldous (1950) found that in *Escherichia coli* the toxic effect of nickel, cobolt, cadmium, zinc and manganese reduced with increased concentration of magnesium in the growth medium. In *Bacillus licheniformis*, Haavik (1976), showed that an addition of 1g l^{-1} magnesium to inhibitory level of manganese, iron, cobolt, nickel and copper could antagonise the effect of these metals.

Say and Whitton (1977) reported that magnesium and calcium reduced Zn toxicity to *Hormidium rivulare*. The influence of calcium increased over a wide range of concentrations compared with magnesium which was more effective in reducing zinc toxicity at lower levels. With zinc sensitive strains of *H. rivulare*, the influence of magnesium was small as was seen in another green alga, *Stigeoclonium tenue* examined by Harding and Whitton (1977). Gächter (1976) however found that the concentration of calcium did not appear to affect the toxicity of zinc, lead, mercury or copper to natural populations of phytoplanktons. The situation is a complex one in that antagonism and synergism may not only occur between heavy metals and essential elements but among themselves (Jones, 1964). Stratton and Corke (1979) found that the combination of Hg ll and Cd or Ni and Cd brought about either a synergistic or antagonistic effect on growth in *Anabaena inaequalis*, depending on the actual combination of metals used. Using sublethal concentration of metals in

pairs, Hg II and Cd brought about a synergistic effect while Cd and Ni an antagonistic effect on growth. The combined effect of zinc and cadmium increased toxicity in *Hormidium rivulare* (Say and Whitton, 1979), which is similar to the effect of these metals to *Lemna valdiviana* (Hutchinson and Czryska, 1972). Hutchinson (1973) reported that copper and nickel interacted synergistically towards growth of some green algae while Se antagonised Cd toxicity. With *Euglena gracilis*, Nakano *et al.*,(1978) found that Cd and Zn antagonised each other. They reported that in the presence of 20 mg l^{-1} Cd the generation time in zinc free medium was 57 hours compared to 27 hours with the addition of 2 mg l^{-1} zinc. Falchuk *et al.*, (1975) found similar results with *Euglena gracilis* in that zinc reduced cadmium toxicity. This is further confirmed by the work of Paklane *et al.*,(1970) and Upitis *et al.*, (1973), who reported similar results with *Chlorella* in that zinc reduced cadmium toxicity. Reporting on a study of the effect of phosphate and nitrate on a blue green alga *Plectonema boryanum*, and a green alga *Chlorella vulgaris*, Rana and Kumar (1974) concluded that the presence of relatively higher concentration of phosphate but not nitrate improve the growth, protecting the algae from the toxic effect of zinc. In *Hormidium rivulare* (Say and Whitton, 1977) and in *Stigeoclonium tenue* (Harding and Whitton 1977), phosphate reduced Zn toxicity. With sulphate however, Say and Whitton (1977) found no detectable influence in reducing zinc toxicity in *Hormidium rivulare*.

1.9 Aims

Literature search indicated that

1. acid stream water differs from site to site both in their mineral content and composition;
2. some cations (Mg, Ca) reduced toxicity to zinc in some algae;
3. metals may react among themselves thus masking their true nature;
4. pH influence toxicity to heavy metals.

It was hypothesised that tolerance to heavy metals in acid stream algae was linked to the nutrient status and the interactions between different metals in solution.

Two specific aims of this project were

(a) to obtain a number of axenic algal strains from acid sites of known water chemistry without the use of any antibiotics, and

(b) to use a common acid stream isolate, *Euglena mutabilis*, to investigate its nutritional requirements and tolerance to heavy metals under standard laboratory conditions.

It was hoped that the result of the investigation would shed some light on the survival strategy of acid stream algae.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Culture Techniques

2.11 Cleaning of Glassware

All glassware (Pyrex brand) was detergent washed and soaked in 2% HNO_3 for over 30 minutes. It was then rinsed in distilled water at least six times and dried in the oven at 100°C before use.

2.12 Culture Vessels

Stock cultures for use as inoculum were maintained in 100 ml straight neck conical flasks closed with silicon bungs. 100 ml Erlenmyer conical flasks with high grade non absorbent cotton wool was used for experiments.

2.13 Sterilization

20 ml of growth medium in 100 ml conical flasks were sterilised by autoclaving at 121°C (10.35 KN m^{-2}) for 15 minutes. The autoclaved medium was left to stand at room temperature for 24 hours to equilibrate with the atmosphere before inoculation.

2.14 Production of Axenic Cultures

As antibiotics induce mutation (Ladha and Kumar 1978) only physical methods were used in isolation and purification. Cells were first grown on solid medium and by repeated transfer of cells from one agar plate to another fungus free colonies were established. This was achieved as follows. Pasteur pipettes were drawn into fine capillary and the end was then heated to form a 'bulb'. The petri dish containing the

algal colonies were examined with a binocular dissection microscope. A few cells from a single colony were removed by placing the 'bulb' of the Pasteur pipette carefully on the colony and streaking on to sterile agar.

Having produced a fungus free culture the next stage in the purification was to eliminate the bacteria. The fungus free cultures were transferred to liquid medium and when the cells have multiplied the liquid culture was sprayed onto sterile agar plates by placing a few drops of culture on a sterile injection needle fixed to a Swinnex filter holder with a sterile 0.2 μm Nuclepore filter. The Swinnex filter holder was connected to a gas cylinder by means of a rubber tubing. When the air was released from the gas cylinder it passed through the sterile filter and forced the algae to spread out in the spray. The spray was caught on agar plates. The plates were covered with cling film and left in the growth room. When colonies appeared they were examined with the dissection microscope and if bacteria were seen then the process was repeated.

2.15 Test for purity

Cells from colonies that appeared to be free of bacteria were transferred to liquid medium and when they multiplied, a few drops of the culture were examined under the microscope. If no bacteria were seen then the cells were placed on different bacterial test media (Table 2.1). These bacterial test plates were covered with aluminium foil to prevent light entering and to encourage bacterial growth, were left in the growth room at 25°C. If no bacterial colonies appeared in 2 weeks of incubation (growth of bacterial colonies in contaminated

agar plates usually occur in 3 days of incubation) the alga was regarded axenic.

TABLE 2.1 Bacterial test media

1. N.A. (Nutrient agar)

   13 g of Nutrient agar (Oxoid, U.K.).

   10 g of Agar (Difco Bacto, Michigan, U.S.A.) in 1000 ml of distilled water.

2. SST

   10 g of glucose

   10 g of tryptone (Oxoid Batch No. 328-6449, U.K.).

   0.5 g of yeast extract (Oxoid, U.K.).

   10 g of agar in 1000 ml of distilled water.

3. P.G. (Peptone and Glucose)

   1.0 g of glucose

   1.0 g of peptone (BDH).

   10 g of Agar in 1000 ml of distilled water.

4. Y.E. (Yeast extract)

   30 g of yeast extract (Oxoid, U.K.).

   10 g Agar in 1000 ml of distilled water.

5. C.A. (Casamino Acid)

   0.12% Casamino acid (Difco Lab.U.S.A.) in the basal growth medium.

   10 Agar.

2.16 Maintenance of Stock Cultures

Axenic stocks of algae (Table 2.2) were maintained on agar slopes in test tubes closed with silicon bungs and incubated in a refrigerator at 7°C under continuous low light. Low light and low temperature will ensure slow rate of cell division and as such individual slopes could be maintained this way for over six months. Fresh slopes were made every six months. All isolates were maintained in the basal medium with added ammonium chloride and 5 mg l^{-1} aluminium, except *Euglena mutabilis* strain D641 isolated from a field zinc concentration of 21 mg l^{-1} was maintained with 10 mg l^{-1} Zn so that it may not lose its tolerance to Zn.

2.17 Sub culturing of Algae

For subculturing of the algae a horizontal laminar flow sterile cabinet (Microflow Pathfinder, conforming to S.B. 5295 Class 1) was used. The cabinet was sprayed with absolute alcohol and the fan switched on and left for about 10 minutes before commencing inoculation. The inoculum was transferred to the culture vessel by using a sterile plastic tip fixed to a Gilson adjustable volume pipetteman. The purity of the inoculum (axenic state) was checked for each experiment using a range of bacterial test media. Periodic checks were made to see if the filters in the inoculating cabinet were functioning properly by placing bacterial test plates in different locations in the cabinet and incubating them in the growth room at 25°C.

TABLE 2.2 List of Algal Stocks

Organism	Durham Culture No.	Stream & Reach No. and country of origin.	State of purity: Clonal	State of purity: axenic
1. *Hormidium* sp.	D451	9001-01 Wyoming, U.S.A.	+	
2. *Hormidium* sp.	D452	9001-01 " "	+	
3. *Chlamydomonas*	D454	9009-20 " "	+	+
4. *Chlamydomonas*	D455	9008-50 " "	+	+
5. *Chlamydomonas*	D456	9013-03 " "	+	+
6. *Chlamydomonas*	D457	0127-05 Brandon Acid Stream,U.K.	+	+
7. *Stichococcus* sp.	D460	0127-05 " " " "	+	+
8. *Chlorella* sp.	D461	9008-50 Wyoming, U.S.A.	+	+
9. *Stichococcus* sp.	D462	9008-50 " "	+	
10. *Eunotia* sp.	D463	0127-05 Brandon Acid Stream,U.K.	+	
11. *Euglena mutabilis*	D464	0127-01 " " " "	+	+
12. *Phormidium* sp.	D472	1005 Belgium		
13. *Raphidonema* sp.	D477	0218 Wales, U.K.	+	
14. *Stichococcus* sp.	D478	9011-01 Wyoming, U.S.A.	+	+
15. *Stichococcus* sp.	D479	0216-15 Wales, U.K.	+	+
16. *Euglena mutabilis*	D640	0221-01 Wales, U.K.	+	+
17. *Euglena mutabilis*	D641	0298 Gategill Mine, England	+	+
18. *Euglena mutabilis*	D642	near Malham Tarn "	+	+
19. *Chlorella* sp.	D643	Mt. Erebus	+	+
20. *Chlorella* sp.	D644	Lake Fudoike, Japan	+	+
21. *Cyanidium caldarium*	D645	" " "	+	+
22. *Pinnularia braunii*	D646	" " "	+	

2.2 Media

2.21 Development of growth Media

For algal growth a modification of No.10 formula of Chu (1942) was used. This was done by reducing carbonate and phosphate and adding EDTA as a chelating agent with trace metals added as AC microelements. As repeated subculture of different strains of *Euglena mutabilis* in nitrates as nitrogen source did not induce its utilization, nitrogen was added as ammonium (NH_4Cl). This modification was used in all algal assays (Table 2.3a; Appendix 1). All stocks except Na_2SiO_3 were of Analar grade and were prepared in de-ionised double distilled water and were stored in a refrigerator (*i.e.* in the dark) at $4^{o}C$. The growth medium (Table 2.1) was prepared in de-ionised double distilled water and the pH was reduced before autoclaving by adding a few drops of 10% H_2SO_4.

2.22 Solid Media

In the preparation of solid media for isolation, purification and storage of algal cultures from acid environments, high quality agar, supplied by Difco Bacto, Michigan, U.S.A., was used. At the initial stages of purification of contaminated algae, 'Pyrex' brand glass petri dishes were used and were replaced with transparent disposable sterile plastic petri dishes. This enables examination without exposure of petri dishes thus avoiding cross contamination.

Agar gets denatured when autoclaved in the presence of acid and does not set on cooling. This was overcome by autoclaving the agar separately and mixing it with double

TABLE 2.3a Composition of growth medium; modifications to Chu's No. 10 formula (Salts)

Salt (mg l^{-1})	Chu's original formula No.10	modified growth medium
K_2HPO_4	10 - 5	-
KH_2PO_4	-	8.0
$MgSO_4$	25	25.0
Na_2CO_3	20	-
Na_2SiO_3	25	25.0
Na_2HCO_3	-	16.0
$CaCl_2$	-	37.0
$Ca(NO_3)_2$	40	-
$FeCl_3$	8	2.24
$MnCl_2$	-	0.45
NaMoO	-	0.007
$MnCl_2$	-	0.05
$ZnSO_4$	-	0.056
$CuSO_4$	-	0.019
$CoSO_4$	-	0.01
HBo_3	-	0.07
NH_4Cl	-	16.0
EDTA (ethylenediaminetetra-acetic acid)		2.45

TABLE 2.3b Composition of the growth medium (elements)

Elements	Composition (mg l^{-1})
N	6.83
P	1.76
S	80
Cl	34.7
Na	7.0
K	2.24
Mg	2.25
Ca	9.77
Si	2.5
Fe	0.5
Mn	0.012
Mo	0.0025
Zn	0.012
Cu	0.005
Co	0.002
B	0.125

strength autoclaved low pH medium while still warm. When agar solidifies, there is an upward shift in the pH by about 0.5 pH units. As such it was necessary to reduce the pH of the medium by a further 0.5 pH units below the required pH value, so that when the autoclaved agar and the medium are mixed together the required pH is achieved. Depending on the humidity in the sterile cabinet (Section 2.17) condensation does take place and this can be avoided by not closing the lids of the petri dishes immediately after pouring the agar.

2.23 Organic Substrates

All organic substrates (Table 2.4) were filter sterilised by passing through a sterile 0.2 µm Nuclepore filter held in an autoclaved Swinnex filter holder and added to the growth medium, aceptically.

2.24 Materials used in toxicity test

The effect of a number of heavy metals (Table 2.5) on growth in *Euglena mutabilis* and the combined effect of Zn and other other heavy metals was checked using stocks of heavy metals prepared in de-ionised distilled water and stored at $4^{o}C$ in the refrigerator. Microelement stocks were prepared omitting the particular element under investigation and added to the growth medium, where applicable.

2.25 pH buffers

For experiments conducted at and below pH 3.0 no buffer was added as there was no change in the pH of the medium even after 30 days of incubation in the basal medium. Whereas for experiments at pH 3.4 and above different buffers

TABLE 2.4 Organic substrates and their concentration in the growth medium

As carbon source	Concentration
D-Glucose	0.01 M
D(-)Fructose	0.01 M
Sucrose	0.01 M
Glycerol	0.01 M
Lactic acid	0.01 M
Acetate (sodium)	0.001 M
As nitrogen source	
β-Alanine	0.01 M
Dl-Asparagine	0.01 M
Glycine	0.01 M
Glycyl-glycine	0.01 M
Urea	0.01 M
Uric acid	0.01 M
Ethanolamine	0.01 M
Phosphorus	
Sodium β-glycerophosphate (BCH product	1.76 mg l^{-1}
DNA (Deoxyriboneuclic acid) sodium salt from Herring sperm type VII, Sigma product	1.76 "
Phytic acid (Inositol Hexaphosphoric acid) Soduum salt from corn type V, Sigma product	1.76 "
Lecithin (Phosphataylcholine) BDH product	1.76 "

TABLE 2.5 List of elements used in toxicity test

Elements tested	Salt used	salt in the basal medium	complementary salts added if element is omitted
Na	$NaNO_3$; NaCl	$NaHCO_3$; $NaSiO_3$	$CaCO_3$; $CaSiO_3$
K	KNO_3	KH_2PO_4	NaH_2PO_4
Mg	$MgSO_4$; $MgCl_2$	$MgSO_4$	---
Ca	$Ca(NO_3)_2$	$CaCl_2$	HCl
Mn	$MnCl_2$	$MnCl_2$	---
Fe	$FeNO_3$	$FeCl_2$	HCl
Al	$AlSO_4$- $AlCl_2$	---	
Zn	$ZnSo_4$- $ZnCl_2$	$ZnSO_4$	HCl
Cu	$CuSO_4$	$CuSO_4$	
Ni	$NiSO_4$- $NiCl_2$	---	
Co	$CoSO_4$	$CoSO_4$	
Pb	$Pb(NO_3)_3$	---	
Cd	$CdCl_2$	---	
N	$NH_4(NO_3)_2$	NH_4Cl	HCl
P	KH_2PO_4	KH_2PO_4	KNO_3

(Table 2.6) were added to the basal medium because the algae were able to reduce the pH of unbuffered medium. To measure the pH of both solid and liquid media model EIL 7050 pH meter was used. When adding different buffers, the required pH value was obtained by adding a few drops of 0.1 M NaOH.

TABLE 2.6 List of pH buffers and their stability range

Buffer	concentration	stability range
Dimethyl glutaric acid	0.5 g l^{-1}	pH 3.2 to 7.0
HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid)	0.5 g l^{-1}	6.8 to 8.2
Borax (sodium tetraborate)	0.5 g l^{-1}	9.0 10.0

2.3 Algal Cultures

2.31 Description of collection sites

2.311 Durham Area

Brandon Acid Stream (Grid Ref. ZN 212 404) is about 5 kilometers from the Science Site of Durham University. It appears to originate from a spoil tip. Hargreaves (1977) hypothesised its origin to be a deep spring in the coal seam. The current proposal from the National Coal Board to operate an opencast mine adjacent to the stream leads to a similar speculation. Whatever be its origin, this stream had been running at a pH of 2.6 for well over a decade. Brandon Pithouse Acid Stream, in addition to its low pH, has a number of heavy metals in solution (Table 2.7). The dominant flora of the stream where it emerges from a clay pipe is the moss

TABLE 2.7 Water chemistry of sites from which *Euglena mutabilis* was isolated (mg l^{-1})

Stream	Grid Ref.	pH	Na	K	Mg	Ca	Zn	Cu	Mn	Fe	Al	Pb	Cd	Ni	Co
Brandon Acid Stream (Strain D 464)	ZN 212 404	2.6	11.7	0.8	52.5	53	1.1	0.57	5.6	82	25	0.1	-	0.5	0.2
Gatehill Mine Stream (Strain D 641)	NY 326 259	3.7	-	-	-	9.6	21.1	0.008	2.42	.26	-	0.41	0.03	0.27	0.1
Adeer Factory effluent (Strain D 640)	NS 298 396	1.9	31.3	3.4	4.5	16.5	0.21	0.083	0.119	6	2	4.9	0.001	0.07	0.05
Nr. Malham Tarn (Strain D 642)		3.5	-	-	-	-	-	-	-	-	-	-	-	-	-

- = no measurement made

Dicranella sp., mostly in the protonemal stage. Other algal assemblage of this stream are *Chlamydomonas applanata* var. *acidophila*, *Euglena mutabilis* and *Eunotia exigua*, with *Euglena mutabilis* on soft mud where the water was flowing very slow.

2.312 Cumbria

Gategill Mine Stream (Grid Ref. NY 326 259) from acid discharge from underground workings of old lead mine had *Englena mutabilis* growing on *Jungermannia* leaves. Chemical analysis of the water showed high concentration of Zn (Table 2.7).

2.313 North Yorkshire

Allan Pentecost provided *Sphagnum palustre* from a site with low pH near Malham Tarn in North Yorkshire Fen. *Euglena mutabilis* was removed from the moss by squeezing the moss and plating it on to agar.

2.314 Other Areas

Stock cultures of acid algae were provided by B.A. Whitton from stocks held at Durham University algal culture collection.

2.32 Morphology of *Euglena mutabilis*

Euglena mutabilis conforms to the general description of euglenoids in that it has a flexible pelicle and a conspecuous red eye spot. One striking difference is that it has no emergent flagellum. Although this characteristic is also found in other *Euglena* species like *E. obtusa*, *E. elenkinii*, *E. fenestrata*, *E. salina* and *E. vermiformis*, *E. mutabilis* can be identified by the presence of two or three large chloroplasts

a number of short rectangular paramylum granules with 5-7 discoid chromataphores. Under different nutritional conditions (heterotrophic growth) lipids are also seen. Electron microphotographs show the characteristic serrated pelicle and the rudiments of the flagellum. Microscopic examination carried out under identical conditions of *E. mutabilis* cells in their exponential growth phase indicated that all four strains of *E. mutabilis* used in this study had its own average size (Table 2.8).

TABLE 2.8 Cell dimensions in fully extended cells of *Euglena mutabilis* from 20 day old cultures in the basal medium

Strain	length when fully extended (observation based on 100 cells)	
D 464	range 60-140 µm	majority 70 - 90 µm
D 460	" 40- 80 µm	" 56 - 64 µm
D 461	" 32- 70 µm	" 40 - 56 µm
D 642	" 40- 90 µm	" 45 - 70 µm
	width when fully extended	
D 464	" 20- 40	majority 25 - 30 µm
D 640	" 10- 30	" 15 - 25 µm
D 641	" 15 - 30	" 20 - 35 µm
D 642	" 20 - 35	" 25 - 30 µm

2.4 Algal Assay

2.41 General

The task of purification of acid stream algae without the use of antibiotics was a very long and time consuming exercise. In all 16 algal strains were obtained (Table 2.7). As much time had been spent in the process of

purification, it was decided to concentrate on one species. *Euglena mutabilis* was chosen because of its wide spread occurrence in acid environments (Section 1.63). For algal assay three strains of *Euglena mutabilis* from three sites of known water chemistry (Table 2.7) were selected. A fourth strain (D642) was included for selected experiments only.

2.42 Incubation and light source

Flasks containing the inoculum were left in the 25°C growth room, on glass shelves and light was provided by warm white fluorescent tubes. The flasks were placed in such a manner as to receive a photon flux density of 100 μmol $m^{-2}s^{-1}$ as measured with the Biospherical Instrument Inc. No. QSP 170 with the laboratory sensor (QSL 100P). In order to provide equal illumination, the flasks were moved about every 24 hours. As the experiments were conducted static, the flasks were shaken at least twice daily.

For the experiment to determine the effect of light and temperature on growth, the temperature cross gradient, an instrument similar to the one described by Van Baalen and Edwards (1973) was used.

In order to prevent light entering the flasks during dark heterotropic growth, the flasks were first wrapped with black polythene sheet and then covered over with aluminium foil. The mouth of the flasks were covered with cotton wool to prevent light entering and not air. The experiment was carried out static in the growth room at 25°C. After an incubation period of 2 months, growth was measured as yield and microscopic examination made for morphological changes.

2.43 Growth medium

Experiments were carried out in 100 ml conical flasks using 20 ml of growth medium. The mouth of the flask was covered with high grade non absorbent cotton wool.

2.44 Inoculum

A standard inoculum judged both by cell number and age structure is important in interpreting the results. In order to achieve this for all the experiments, only cells of 10 to 15 day old at their exponential growth phase was used. The stock culture for inoculum was diluted to get a standard inoculum size of 100 cell ml (±15) for each experiment.

2.45 Cell count and doubling time

At the end of an experiment, the cell density was estimated by counting the number of cells in each flask. This was done by taking 0.05 ml of culture on to a haemacytometer and counting all the cells on the grid. Four samples were taken from each flask and as each experimental condition had four replicates, the result is that of 16 separate cell counts.

Growth rate as doubling time was calculated by subtracting the number of cells at the end of the experimental period from that of the beginning and dividing the result by the time using the formula of Fogg (1975)

$$K^1 = \frac{\log_{10} n - \log_{10} N^o}{t}$$

$$\text{Generation time (G)} = \frac{0.301}{K^1}$$

where K^1= Relative growth constant

N = Number of cells at the end of the experiment

N^{O} = Number of cells at the beginning of the experiment

t = Time

G = Generation time (doubling time)

2.46 Dry weight

At the end of an experiment the dry weight of the cells was taken as mg ml^{-1}. One ml of culture was removed from each flask with a Gilson adjustable volume pipetteman and placed on a 2.5 cm GF/C filter (Whatman U.K.) and was filtered under suction. The cells were washed four times with distilled water to prevent any carry over of materials from the growth medium. Each filter paper containing the algae was folded once to form a semi circle and placed on separate aluminium foil to avoid filters sticking together. The filters were dried in the oven at 105°C for 48 hours. At the end of 48 hours the filters in their aluminium foil covers were quickly transferred to a desiccator to prevent absorption of water as they cooled to ambient temperature. The filters were weighed in a Mettler type 16 balance. Pre-determined weight of the filter paper (mean of 25 filters from the same batch dried in the oven at 105°C for 48 hours) was subtracted from the weight of the filter with the algal contents to determine the weight of the algal cells.

2.47 Microscopy

Growth of algae on solid media during purification and a test for purity (Section 2.32) were examined using a Nikon binocular microscope. Cell count, and morphology in liquid cultures was carried out on a Carl Zeiss microscope by placing 0.05 ml of culture in a haemacytometer counting

chamber (depth 1 mm; Improved Neuber ruling).

2.5 Quantitative Experiments

2.51 Influence of light and temperature on growth

In order to establish the optimum light intensity and temperature regime for growth of *Euglena mutabilis* under laboratory conditions strain D464 was grown in the basal medium at pH 2.6 for 20 days at a photon flux density of 40, 80, 100 and 120 μmol $m^{-2}s^{-1}$ and temperature regimes of 15, 20, 25 and 30°C in the cross gradient (Section 2.42). There were 4 flasks for each temperature regime and light intensity. At the end of 20 days of incubation, each flask was counted four times for cell number and dry weight was taken (Sections 2.44; 2.45).

2.52 Influence of pH on growth

With a view to finding the optimum pH for growth under laboratory conditions, *Euglena mutabilis* strains D464, D640, D641 and D642 were subcultured in a series of pH values ranging from pH 1.0 to 10, using inocula from stocks grown in the field pH values (Table 2.7). Experiments were carried out static in the growth room at 25°C and a photon flux density of 100 μmol $m^{-2}s^{-1}$ for 20 days. The experiment was repeated using inocula from a standard pH of 2.6 with strains D464, D640 and D641. In order to see if long term subculture in low and high pH could bring about adaptation, *E. mutabilis* D464 was subcultured for 6 months in pH 1.8 and 7.6, the experiment was repeated using inocula from pH 1.8 and 7.6 with inocula from pH 2.6 as control over the same range of pH values.

2.53 Exposure and survival time at extreme pH values

In order to see whether cells exposed to pH 1.0, 1.2, 1.4 and 10.0 (where cells looked dead) were viable, they were removed from the flasks by filtering on a 0.25 cm GF/C filter and were placed in the basal medium at pH 2.6. Their recovery from exposure to specific pH on a time scale from 30 minutes to 20 days of exposure was carried out, under standard growth condition.

2.54 Influence of macro and micro nutrients on growth in *Euglena mutabilis*

In order to check the effect of macronutrients (Na, K, Mg, Ca) and micronutrients (Zn, Cu, Co, Mn) on growth in *E. mutabilis*, strain D464, D640 and D641, modifications to the basal medium were made as follows. Microelement stocks were prepared omitting the particular element under investigation. With macroelements, sodium present in the basal medium as salts of carbonate and silicate were replaced with sodium nitrate. This resulted in an increase of Na concentration from 7 to 10 mg l^{-1}. With experiments with potassium, K was added as KNO_3 replacing KH_2PO_4 with sodium salt with the same concentration of PO_4-P. Inocula for experiments to check the effect of macronutrients, was taken from the basal medium where as for experiments dealing with micronutrients, was taken from stocks grown in the absence of the particular element under investigation. Using a standard inoculum of 10^2 cells ml^{-1} growth as yield was checked after 20 days of incubation under standard growth conditions (Section 2.42). Each experiment was repeated four times with four replicates.

2.55 Influence of vitamins on growth

In order to see the effect of vitamin B_1 and B_{12} on yield in *Euglena mutabilis*, strain D464 was subcultured in the basal medium in the presence of 1 µg l^{-1} vitamin B_1 and 1 µg l^{-1} vitamin B_{12} and combined vitamin B_1 with B_{12} with vitamin free basal medium as control, under standard growth conditions (Section 2.4). Vitamins were sterile filtered and added aseptically. As cobalt forms an important constituent of vitamin B_{12} it was hypothesised that an increase in the cobalt concentration (Table 2.9a) could bring about the same effect as vitamin B_{12}. In order to check this hypothesis stocks were grown in cobalt free medium and cells were transferred to growth medium with different concentrations of Co. The growth as yield was measured after 20 days of incubation.

2.56 Utilization of organic substrates in light and in darkness

With a view to see if *Euglena mutabilis* could utilize different organic substrates for growth and reproduction in the presence and absence of light, investigations were carried out using strains D464, D640 and D641 under standard growth conditions (Section 2.42). The organic substrates (Table 2.4) were added aseptically to the autoclaved growth medium and growth as yield was measured as cell number and dry weight.

2.57 Utilization of organic phosphates

2.571 Organic P determination

Phosphorus determination to establish whether organic P was stable at pH 2.6 at which pH growth experiments were conducted, was carried out according to the

TABLE 2.9a List of Microelements and their concentrations used in the growth experiments (mg l^{-1})

Con.	0.001	0.002	0.005	0.013	0.025	0.05	0.1	0.2	0.25	0.4	0.5	0.6	0.8	1.0	1.5	2.0	4.0	6.0	8.0	10.0
Zn	✓			✓		✓					✓			✓		✓	✓	✓	✓	✓
Cu	✓	✓	✓	✓		✓					✓	✓	✓	✓						
Mn	✓			✓	✓	✓	✓				✓			✓						
Co	✓	✓					✓		✓		✓			✓						

TABLE 2.9b List of Macroelements and their concentrations used in growth experiments (mg l^{-1})

Con.	0	2.5	5.0	7.0	10.0	20.0	40.0	80	120	140	160	220
Na	-	-	-	✓	✓	✓	✓	✓	✓	✓	✓	
K	-	✓	✓		✓	✓	✓	✓	✓			
Mg		✓	✓		✓	✓	✓	✓			✓	
Ca			✓		✓	✓	✓	✓	✓		✓	✓

modifications of Eisenreich *et al.*, (1975).

2.572 Soluble reactive phosphorus (SRP)

An aliquot of organic P solution (Table 2.2) was diluted to 25 ml with distilled water. Then 5 ml of 'mixed reagent' was added. Colour development was allowed to proceed for 10 minutes. Then absorbance was read at 880 nm on the Shimadzu Double Beam Spectrophotometer. The P concentration was determined using a calibration curve.

2.573 Total phosphorus (TP)

This was determined as follows. 5 ml of 1N H_2SO_4 was added to 100 ml of organic P solution in 250 ml Erlenmyer flask followed by addition of 0.7g potassium persulphate. The flask was covered with aluminium foil and autoclaved at 121^{o}C for 30 minutes. After cooling, 5 ml of 'mixed reagent' was added and colour development was allowed to proceed for 10 minutes. The absorbance was read at 880nm on the Shimadzu Double Beam Spectrophotometer. The P concentration was determined using a calibration curve constructed in the same way as for SRP determination.

2.574 Acid phosphatase activity

Growth experiments at different pH regimes in which growth was seen both at acid and alkaline pH values suggested that *Euglena mutabilis* may have both acid and alkaline phosphatase activities as a strategy for growth both at acid and alkaline pH. In order to investigate phosphatase activity, 20 day old cultures of *Euglena mutabilis* D464 was used. Cells were filtered through a 0.45 membrane filter and transferred to acid washed Universal bottles.

2 ml of acetate buffer (pH 5.0) was added, followed by 0.5 ml of 0.0. M $CaCl_2$. The Universal bottle was left to equilibrate at 25°C for 15 minutes in the water bath. Then 0.5 ml of phosphate substrate (p-nitrophenyl phosphate) was added and left to incubate in the water bath for 15 minutes. 8 ml of NaOH 0.05 N was added. The contents were mixed thoroughly and filtered. The absorbance was then read at 410 nm on Shimadzu Double Beam Spectrophotometer.

2.575 Alkaline phosphatase activity

For alkaline phosphatase activity instead of acetate buffer, Tris buffer (pH 9.0) was added and the rest of the procedure followed as for acid phosphatase and the absorbance read using the Shimadzu Double Beam Spectrophotometer at 410 nm.

2.58 Heavy metal tolerance

One of the significant features of acid streams is the presence of heavy metals (Section 1.4). As *Euglena mutabilis*, strains D464, D640 and D641 were isolated from streams with a number of heavy metals (Table 2.6), it was decided to investigate the tolerance to Zn, Cu, Mn, Fe, Al, Pb, Cd, Ni and Co in the basal growth medium at pH 2.6 (Table 2.10). Metal stocks were prepared in de-ionised double distilled water from which different concentrations were added to the growth medium. A standard inocula of 10^{-2} cells ml^{-1} was incubated in different metal concentrations under standard growth conditions (Section 2.42).

When any particular element was omitted, complementary salts were added (Table 2.5) replacing those in the basal medium. Each experiment was carried out four times

with four replicates. At the end of the incubation period of 20 days, each flask was sampled four times for cell number and the dry weight taken (Section 2.45; 2.46). Microscopic examination (Section 2.47) was carried out to see if any morpholoigal changes in the cells, due to the presence of heavy metals, in the growth media.

2.59 Factors influencing zinc toxicity in *Euglena mutabilis* D464

2.591 Influence of inoculum size

In order to have a standard inoculum size which will result in a recognisable yield both as cell number and dry weight at the end of 20 days of incubation at sub-lethal (40 mg l^{-1}) Zn, different cell densities of *E. mutabilis* D464 from stocks grown in the basal medium at pH 2.6 were incubated for 20 days. At the end of the period of incubation, each flask was examined four times for cell number and the dry weight recorded. Having repeated the experiment four times with four replicates, it was seen that to have an appreciable yield in 20 days, the inoculum size of 10^{-2} cells ml^{-1} was necessary. Therefore this inoculum size was used for all experiments.

2.592 Influence of major cations and anions on zinc toxicity

The influence of major cations and anions (Table 2.10) on Zn toxicity was checked using different concentrations of Na, K, Mg, Ca, Cl and ammonium nitrogen in 20 ml of growth media under standard growth conditions. When certain element was omitted, complementary salts were added to compensate for the loss (Table 2.5). Influence

of Cl on Zn toxicity at sublethal level was checked with HCl. Each experiment was done four times with four replicates. At the end of 20 days of incubation cell count and dry weight measurements were taken (Sections 2.45; 2.46).

2.593 Influence of organic substrates on zinc toxicity

The influence of glycyl-glycine and β-glycerophosphate on yield in *E. mutabilis*, at sub-ethal zinc concentration (40 mg l^{-1}), was investigated using different concentrations of these organic substrates. Stocks of these substrates were prepared in de-ionised double distilled water and made sterile by passing through a sterile 0.2µm Nucleopore filter and added to autoclaved media under sterile conditions. Yield was measured as cell density and dry weight after 20 days of incubation under standard growth conditions.

2.594 Influence of other heavy metals on zinc toxicity

In order to check the influence of other heavy metals (Cu, Mn, Fe, Al, Cd, Ni, Co) on zinc toxicity in sublethal and inhibitory Zn levels, (20 mg l^{-1}) metal stocks were prepared in de-ionised double distilled water from which different concentrations were added to the basal medium (Table 2.10). Each metal was checked four times with four replicates for each concentration. At the end of 20 days of incubation, cell count and dry weight measurements were taken.

TABLE 2.10 List of Elements and their concentrations used in toxicity tests ($mg\ l^{-1}$)

Element→	N*	N†	P·	Cl	Na	K	Ca	Mg	Zn	Cu	Mn	Fe	Al	Pb	Cd	Ni	Co
≤0.0002									✓	✓							
0.002									✓	✓							✓
0.005										✓					✓		
0.01										✓	✓				✓		
0.025									✓								
0.05									✓	✓				✓	✓		
0.1										✓				✓	✓		
0.25														✓	✓	✓	✓
0.5									✓	✓		✓		✓	✓	✓	✓
0.75														✓		✓	
1.0									✓	✓	✓	✓	✓	✓		✓	✓
1.8			✓														
2.5			✓			✓		✓	✓			✓	✓				
5.0	✓		✓			✓	✓	✓	✓		✓	✓	✓	✓		✓	✓
7.5					✓				✓			✓					
10	✓	✓	✓		✓	✓	✓	✓	✓		✓	✓	✓	✓		✓	✓
15					✓												
20	✓	✓	✓		✓	✓	✓	✓	✓		✓		✓	✓		✓	✓
25		✓			✓												
30	✓	✓	✓		✓		✓									✓	
35	✓			✓													
40	✓	✓		✓	✓	✓	✓	✓	✓		✓		✓	✓		✓	✓
50		✓		✓					✓				✓	✓			✓
60		✓		✓													
70		✓		✓													
80		✓		✓	✓	✓	✓	✓			✓		✓				
90				✓													
120		✓															
160		✓			✓	✓	✓	✓					✓				
200								✓					✓				
1000													✓				

* = glycyl-glycine

† = ammonium

· = β-glycerophosphate

CHAPTER THREE

RESULTS

3.1 Introduction

Experiments were designed and carried out under standard growth conditions (Section 2.4) to see the effects of a number of environmental factors on growth in *Euglena mutabilis*. The results are based on experiments repeated four times with four replicated for each parameter under investigation. All experiments were carried out in the growth room at 25°C and a photon flux density of 100 µmol m^{-2} s^{-1}.

3.2 Influence of temperature and light intensity on growth (yield) in *Euglena mutabilis* D464

In order to establish the optimum temperature and light intensity for growth under laboratory conditions, *Euglena mutabilis* D464 was subcultured at different light intensities and temperatures on the cross-gradient apparatus (Section 2.44). The inoculum was taken from the basal medium at pH 2.6. With four temperature regimes and four light intensities, yield was checked over a 20 day period (Figure 3.1, Table 3.1). Optimum yield was at 25°C at a photon flux density of 100 µmol m^{-2} s^{-1}.

Fig. 3.1 Influence of light intensities and temperature regimes on yield in *E. mutabilis* D464 in 20 days using an inoculum of 10^2 cells ml^{-1}

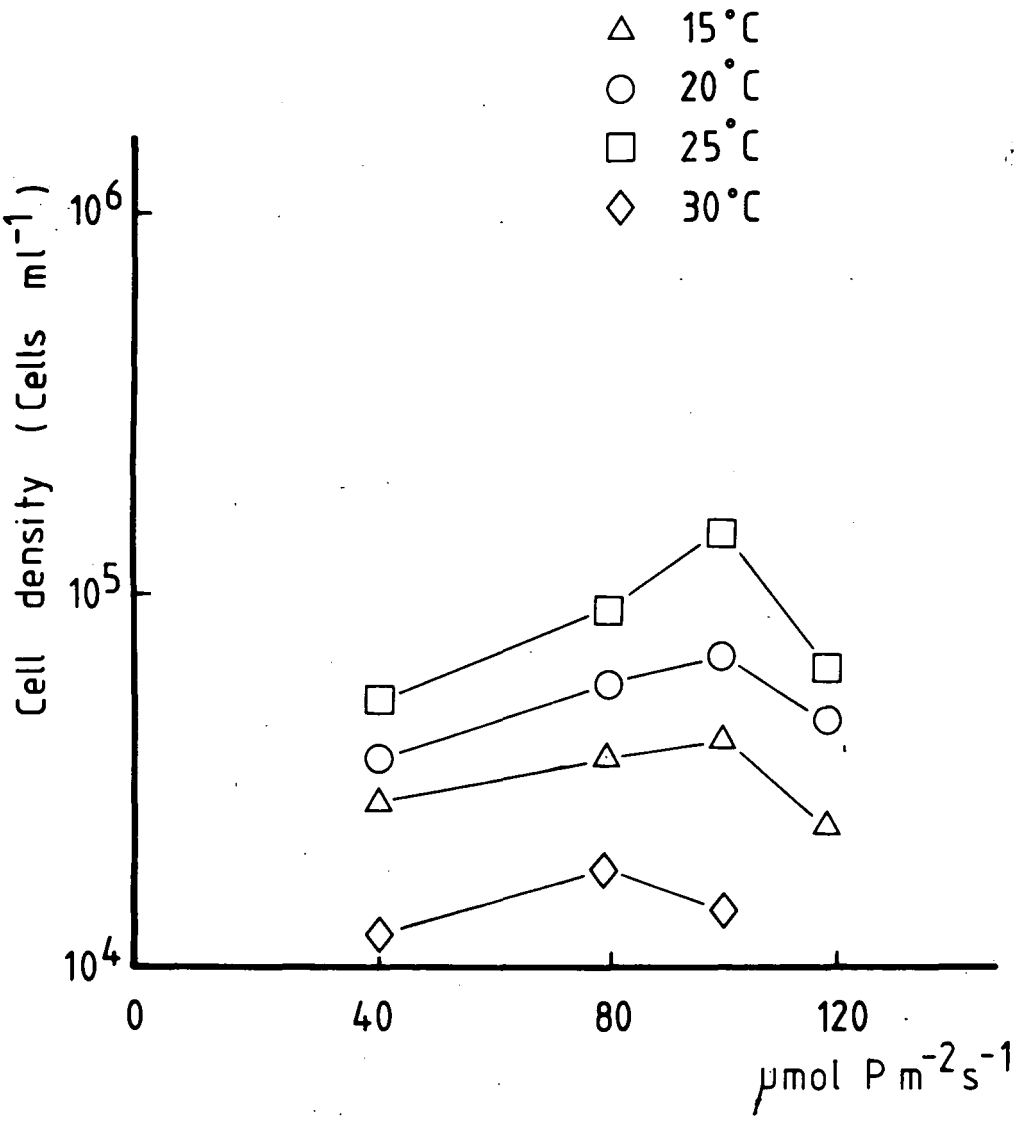

15 °C
20 °C
25 °C
30 °C
Cell density (Cells ml^{-1})
10^6
10^5
10^4
0
40
80
120
µmol P m^{-2}s^{-1}

TABLE 3.1 Influence of different light intensities and light regimes on yield in *Euglena mutabilis* D464 in 20 days using an inoculum of 100 cells ml^{-1}. n = 16; dry weight in $g\ l^{-1}$

Light intensities ($\mu mol\ P\ m^{-2} s^{-1}$)		Temperature regimes (^{o}C)			
		15	20	25	30
40	$\bar{x}$	0.23	0.26	0.39	0.21
	sd	0.05	0.05	0.03	0.03
80	$\bar{x}$	0.26	0.28	0.42	0.24
	sd	0.04	0.07	0.07	0.04
100	$\bar{x}$	0.29	0.33	0.51	0.22
	sd	0.05	0.05	0.01	0.05
120	$\bar{x}$	0.2	0.28	0.38	----
	sd	0.07	0.05	0.03	

3.3 Influence of pH

3.31 Influence of pH on growth

In order to check the influence of pH on growth in *Euglena mutabilis*, inoculum from strains D464, D640, D641 and D642 were produced by culturing at the same pH values as in their natural environments from whence they were isolated (Section 2. ; Table 3.2) and using a standard inoculum (Section 2.43), their growth was checked as yield in a range of pH values from 1.0 to 10.0 after 20 days of incubation (Fig. 3.2). With a view to see the effect of pH on growth with inoculum from a standard pH, strains D464, D640 and D641 were subcultured at pH 2.6 to produce the inoculum and the experiment was repeated (Fig. 3.3). In both experiments the yield was optimum between pH 3.4 and 4.0, (see Table 3.3).

TABLE 3.2 Influence of pH on yield (dry weight) in *E. mutabilis* D464, D640, D641 and D642 using inocula from stocks grown at field pH values. Inoculum size = 100 cells ml^{-1}; n = 16; dry weight in g l^{-1} ; - = no growth

		D464	D640	D641	D642
pH					
1.8	$\bar{x}$	0.42	0.34	0.35	----
	sd	0.01	0.03	0.02	
2.2	$\bar{x}$	0.44	0.84	0.38	----
	sd	0.05	0.07	0.01	
2.6	$\bar{x}$	0.51	1.33	0.42	----
	sd	0.03	0.06	0.05	
3.0	$\bar{x}$	0.87	1.41	0.49	0.80
	sd	0.01	0.1	0.06	0.05
3.4	$\bar{x}$	1.33	1.45	0.91	1.06
	sd	0.06	0.03	0.04	0.01
4.0	$\bar{x}$	1.32	1.43	0.89	1.30
	sd	0.07	0.04	0.02	0.05
5.0	$\bar{x}$	0.29	1.41	0.84	1.24
	sd	0.05	0.01	0.07	0.05
6.0	$\bar{x}$	0.97	0.98	0.49	0.84
	sd	0.01			
6.8	$\bar{x}$		0.82		
	sd		0.07		
7.0	$\bar{x}$	0.74	----	0.43	0.70
	sd	0.01		0.04	0.03
7.6	$\bar{x}$	0.53	----	0.42	
	sd	0.04		0.05	
8.0	$\bar{x}$	----	----	----	0.63
	sd				0.05
8.5	$\bar{x}$	----	----	----	0.54
	sd				0.07

TABLE 3.3 Influence of pH on yield (dry weight) in *E. mutabilis* D464, D640 and D641 using inocula from stocks grown in a standard pH (pH 2.6). Inoculum size = 100 cells ml^{-1}; dry weight in $g\ l^{-1}$; n = 16; ---- = no growth

pH		D464	D640	D641
1.8	$\bar{x}$	0.42	0.38	0.39
	sd	0.03	0.05	0.01
2.2	$\bar{x}$	0.43	0.44	0.41
	sd	0.06	0.01	0.07
2.6	$\bar{x}$	0.51	1.33	0.48
	sd	0.07	0.01	0.06
3.0	$\bar{x}$	0.87	1.42	0.50
	sd	0.01	0.05	0.04
3.4	$\bar{x}$	1.32	1.53	0.51
	sd	0.04	0.06	0.01
4.0	$\bar{x}$	1.31	1.52	0.53
	sd	0.07	0.07	0.02
5.0	$\bar{x}$	1.29	1.41	0.52
	sd	0.08	0.1	0.05
6.0	$\bar{x}$	0.97	1.40	0.52
	sd	0.1	0.05	0.03
6.8	$\bar{x}$	----	0.48	
	sd		0.01	
7.0	$\bar{x}$	0.73	----	0.52
	sd	0.05		0.03
7.6	$\bar{x}$	0.53		0.47
	sd	0.02		0.01

Fig. 3.2 Influence of pH on yield in *E. mutabilis* D464, D640, D641 and D642 with inocula from stocks grown at field pH values in batch culture at 25°C and photon flux density of 100 μmol m^{-2} s^{-1}

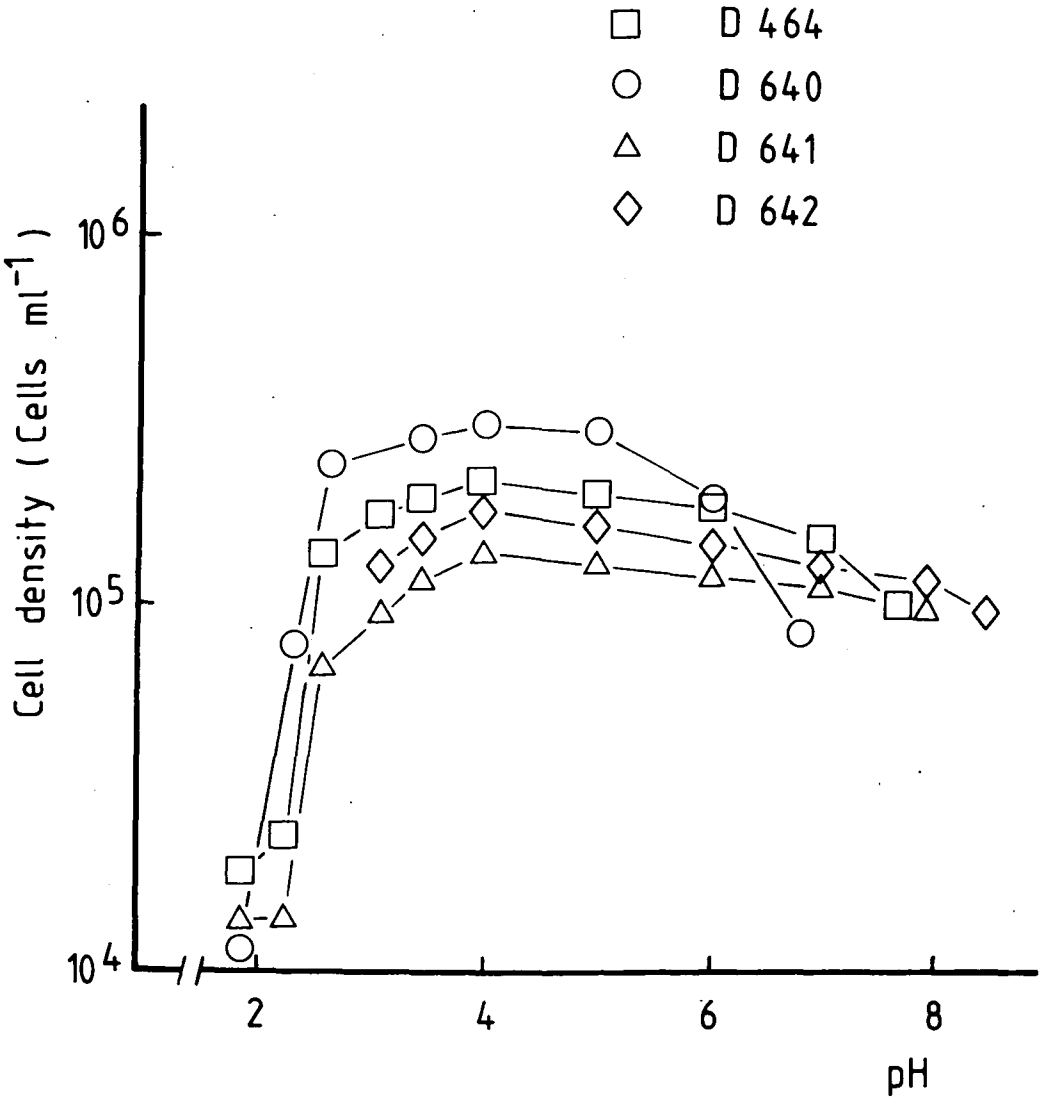
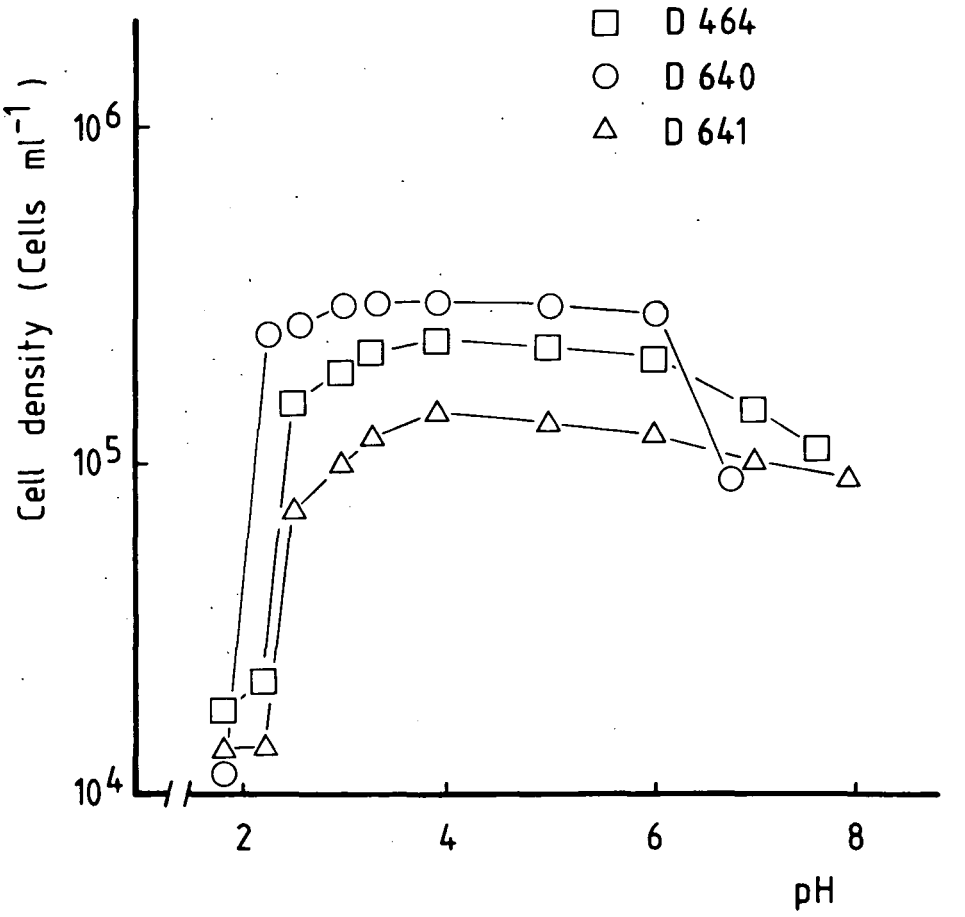
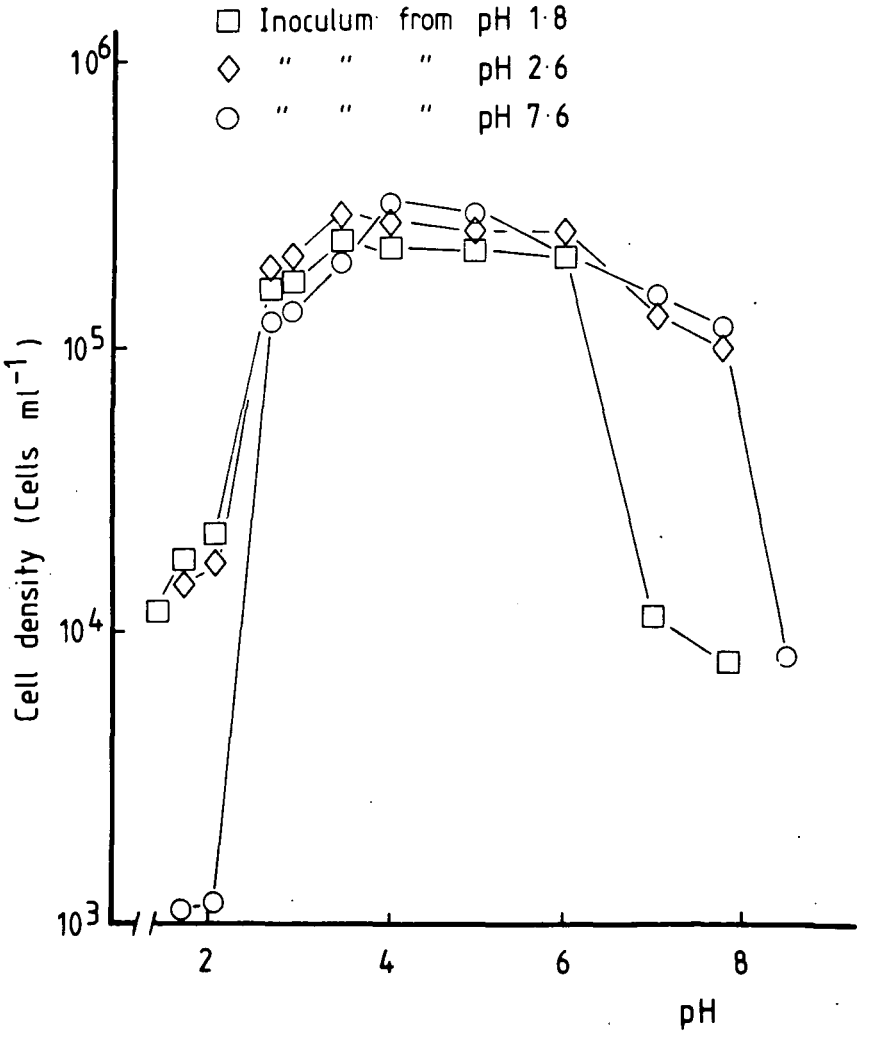

D 464
D 640
D 641
D 642
Cell density (Cells ml^{-1})
10^6
10^5
10^4
2
4
6
8
pH

Fig. 3.3 Influence of pH on yield in *E. mutabilis* D464, D640 and D641 subcultured from a standard pH of 2.6 in batch culture at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}

Fig. 3.4 Influence of prolonged subculture at pH 1.8 and 7.6 on yield at different pH values at 25°C and photon flux density of 100 µmol m^{-2} s^{-1} in *E. mutabilis*


D 464
D 640
D 641
Cell density (Cells ml^{-1})
10^6
10^5
10^4
2
4
6
8
pH


Inoculum from pH 1·8
" " " pH 2·6
" " " pH 7·6
Cell density (Cells ml^{-1})
10^6
10^5
10^4
10^3
2
4
6
8
pH

3.32 Influence of prolonged subculture on adaptation to higher and lower pH values

In order to see if prolonged subculture at pH 1.8 and 7.6 could bring about an adaptation to lower and higher pH, strain D464 was subcultured for six months at these pH values (Section 2.472) and the experiment was repeated. The results (Fig.3.4 and Table 3.4) compared with the results of earlier experiments (Fig.3.3) showed that growth had taken place at pH values hitherto not seen; indicating that adaptation to these pH values had taken place.

3.33 Exposure time and survival at very low pH values

In earlier experiments (Section 3.2) strain D464 when subcultured from pH 1.8 in a series of pH values from 1.0 to 10.0, there was no visible growth in pH 1.0, 1.2 and 10.0 in 20 days of incubation. When these cells were transferred to the basal medium, there was recovery in cells exposed to pH 10.0 and not in pH 1.0 and 1.2. Experiments carried out to see their survival time under these low pH values (Section 2.472) indicated that the maximum exposure time for recovery at pH 1.0 is 6 hours and in pH 1.2, four days (Tables 3.1 and 3.5).

TABLE 3.5 Time course of survival in very low pH values in *Euglena mutabilis* D464

maximum exposure time for recovery	at pH 1.0	at pH 1.2
	6.0 h	4.0 d

TABLE 3.4 Influence of prolonged subculture in low and high pH media on growth in different pH values in *E. mutabilis* D464 measured as dry weight ($g\ l^{-1}$) in 20 days using an inocula of 100 cells ml^{-1}; n = 16.

		Inocula from		
pH		pH 1.8	pH 2.6	pH 7.6
1.5	$\bar{x}$	0.35	------- no growth --------	
	sd	0.01		
1.8	$\bar{x}$	0.42	0.41	0.35
	sd	0.01	0.05	0.05
2.2	$\bar{x}$	0.44	0.51	0.35
	sd	0.04	0.01	0.05
2.6	$\bar{x}$	0.51	0.51	0.39
	sd	0.05	0.07	0.06
3.0	$\bar{x}$	0.83	0.87	0.76
	sd	0.07	0.05	0.08
3.4	$\bar{x}$	1.29	1.31	1.30
	sd	0.1	0.1	0.5
4.0	$\bar{x}$	1.30	1.31	1.31
	sd	0.1	0.2	0.3
5.0	$\bar{x}$	1.27	1.29	1.30
	sd	0.1	1.05	0.04
6.0	$\bar{x}$	0.94	0.97	1.01
	sd	0.02	0.1	0.07
7.0	$\bar{x}$	0.34	0.73	0.92
	sd	0.1	0.06	0.04
7.6	$\bar{x}$	0.22	0.53	
	sd	0.01	0.05	
8.0	$\bar{x}$	no growth		0.87
	sd			0.01
8.5	$\bar{x}$	no growth		0.78
	sd			0.01

3.34 Influence of pH on doubling time in *Euglena mutabilis*

Experiments conducted to check the doubling time in *E. mutabilis*, strains D464, D640 and D641 (Section 2.44), in different pH values indicated (Table 3.6) that although all three strains had different doubling times, the shortest doubling time was seen between pH 3.4 and 4.0 in all strains tested.

3.35 Influence of pH on morphology

Microscopic examination of cells showed at low pH limit of 1.8 over 50% of the cells were rounded up (*c.* 28 µm in dia.) and in most cases these rounded cells were brown in colour. Frequently cells were seen together in pairs. At and below pH 1.5 many of the cells were colourless. There were examples of cells which had retained the fusiform shape of *Euglena* but had a transparent protoplasm.

In the upper pH range at and above pH 6.0 'giant cells' were often seen along with normal cells. They were about the same length as the normal cells (Table 2.8 & 3.7) but were not moving and several times broader with a very compactly packed granular cytoplasm. Another conspicuous feature often seen at the higher pH range was that cells were stuck together in a gelatinous mass. At the optimum pH (pH 3.4-4.0) however, the cells, though granular, were very actively moving about.

TABLE 3.6 Doubling time (h) in *Euglena mutabilis* at 25°C and photon flux density of 100 µmol m^{-2} s^{-1} in batch culture at pH 2.6

pH	0 - 5 days			0 - 10 days			0 - 15 days			0 - 20 days		
	D464	D640	D641	D464	D640	D641	D464	D640	D641	D464	D640	D641
1.8	97.5	30.7	107.1	84.7	34.5	104.	86.1	49.8	104.5	88.2	52.2	103.7
2.2	47.6	30.1	99.2	66.3	32.2	98.7	68.2	39.2	96.5	60.1	48.3	97.2
2.6	42.n	24.7	97.3	52.6	22.5	80.2	60.3	36.3	86.3	66.6	42.7	86.9
3.0	40.4	25.5	97.5	40.5	25.5	80.5	43.2	29.9	81.7	45.1	42.5	80.6
3.4	42.2	25.3	98.1	40.3	27.6	68.1	41.1	29.8	72.5	43.2	42.1	72.1
4.0	47.4	27.5	98.5	41.6	32.5	70.3	42.3	36.8	74.9	43.4	42.3	74.5
5.0	49.0	27.2	99.1	46.2	33.5	81.2	48.1	39.4	79.3	51.2	43.2	82.7
6.0	59.5	29.2	99.8	53.2	35.3	82.3	54.2	39.7	83.4	55.3	44.3	84.3
7.0	-	-	-	67.5	-	97.5	69.3	-	88.6	68.5	-	89.8
8.0	-	-	-	-	-	-	-	-	-	-	-	-

TABLE 3.7 Influence of pH on cell size in *Euglena mutabilis* D464

Cell size	pH 1.8	pH 2.6	pH 3.4	pH 7.6
Range length (µm)	20-40	32-140	40-150	35-120
width (µm)	2-14	2-18	2.5-22	2-20
majority length (µm)	32-36	40-90	45-100	43-80
wiegh (µm)	2.5-9	2.5-12	3-18	2.5-15

3.4 Influence of macronutrients on yield

3.41 Introduction

Experiments were conducted to check the effect of Na, K, Mg and Ca on yield in different strains of *Euglena mutabilis*. Using standard inocula, strains D464, D640 and D641 were subcultured at 25°C and a photon flux density of 100 µE m^{-2} s^{-1} for 20 days. Each experiment was repeated four times with four replicates for each concentration of element under investigation. The basal medium had the following concentrations of macroelements:

Na 7 mg l^{-1}

K 2.5 mg l^{-1}

Mg 2.5 mg l^{-1}

Ca 10.0 mg l^{-1}

3.42 Influence of sodium on yield

Growth measured as yield in *Euglena mutabilis*, D464, D640 and D641 indicated that an increase in the Na concentration up to 25 mg l^{-1} brought about an increase in the cell density. Further increase above 25 mg l^{-1} reduced yield (Fig.3.5, Table 3.8).

Fig. 3.5 Influence of Na on yield in *E. mutabilis* D464, D640 and D641 in batch culture at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}

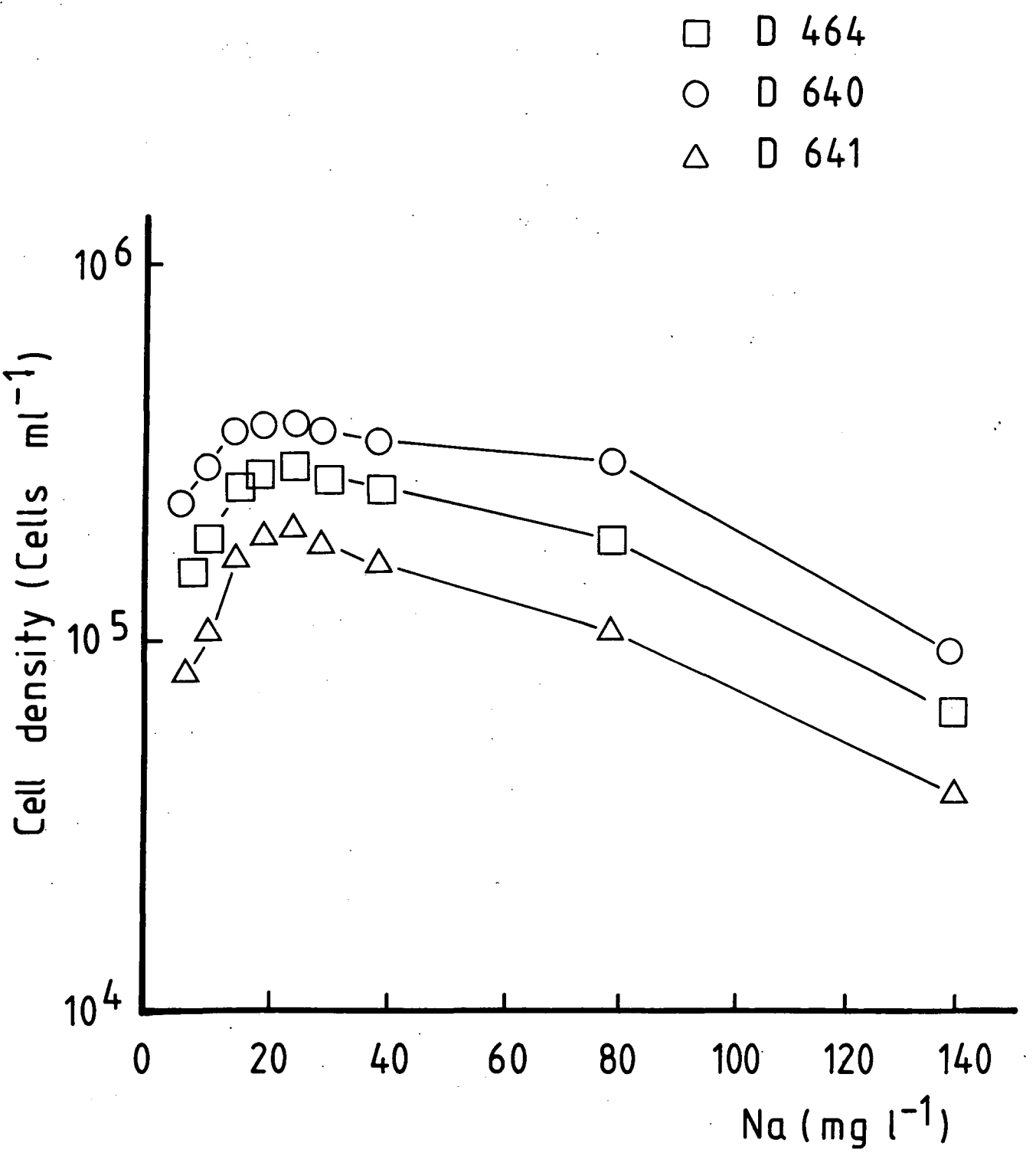

D 464
D 640
D 641
10^6
10^5
10^4
Cell density (Cells ml^{-1})
0
20
40
60
80
100
120
140
Na (mg l^{-1})

TABLE 3.8 Influence of sodium on yield in *E. mutabilis* D464, D640 and D641 measured as dry weight ($g\ l^{-1}$) in 20 days in batch culture in 25°C and photon flux density of 100 $\mu mol\ m^{-2}\ s^{-1}$; n = 16.

Na ($mg\ l^{-1}$)		D464	D640	D641
7.0	$\bar{x}$	0.52	0.94	0.37
	sd	0.01	0.06	0.01
10.0	$\bar{x}$	0.55	1.03	0.41
	sd	0.01	0.1	0.05
15.0	$\bar{x}$	0.63	1.17	0.47
	sd	0.1	0.05	0.1
20.0	$\bar{x}$	0.82	1.29	0.59
	sd	0.1	0.1	0.01
25.0	$\bar{x}$	0.91	1.32	0.63
	sd	0.12	0.15	0.07
30.0	$\bar{x}$	0.78	1.30	0.61
	sd	0.06	0.1	0.01
40.0	$\bar{x}$	0.76	1.29	0.52
	sd	0.1	0.15	0.08
80.0	$\bar{x}$	0.52	0.92	0.44
	sd	0.06	0.03	0.01
140	$\bar{x}$	0.31	0.39	0.29
	sd	0.01	0.05	0.03

3.43 Influence of potassium on yield

The influence of K on yield was similar in that all three strains showed optimum yield at 5.0 mg l^{-1}. Further increase had no effect on yield (Fig.3.6, Table 3.9).

3.44 Influence of magnesium on yield

The influence of Mg on yield both as cell number and dry weight in three strains of *Euglena mutabilis* D464, D640 and D641 were examined under standard growth conditions. Results (Fig.3.7) indicate a requirement for Mg as there was no growth (yield) in the total absence of this element. Increase up to 10 mg l^{-1} brought about an increase in yield and further increase up to and including 160 mg l^{-1} had no effect on yield in all three strains tested (Table 3.10).

3.45 Influence of calcium on yield

The effect of Ca on yield under standard growth conditions (Section 2.44), was checked with three strains of *Euglena mutabilis*. The results (Fig.3.8, Table 3.9) indicated optimum yield at 80 mg l^{-1} Ca. There was no growth in any of the three strains in the absence of Ca (Table 3.11).

3.5 Influence of micronutrients on yield

3.51 Introduction

The influence of major microelements on growth in *Euglena mutabilis* was carried out under standard laboratory conditions (Section 2.4) in the basal medium at pH 2.6. Using a standard inocula of 10^2 cells ml^{-1} from stocks grown in the

TABLE 3.9 Influence of potassium on yield in *E. mutabilis* D464, D640 and D641 in batch culture, measured as dry weight (g l^{-1}) in 20 days in 25°C and photon flux density to 100 µmol m^{-2} s^{-1}. n = 16.

K (mg l^{-1})		D464	D640	D641
1.0	$\bar{x}$	0.21	0.19	0.19
	sd	0.05	0.07	0.04
2.0	$\bar{x}$	0.37	0.29	0.24
	sd	0.04	0.05	0.03
2.5	$\bar{x}$	0.52	0.91	0.39
	sd	0.03	0.05	0.07
5.0	$\bar{x}$	0.53	0.92	0.40
	sd	0.01	0.04	0.02
10.0	$\bar{x}$	0.52	0.93	0.40
	sd	0.01	0.05	0.04
20.0	$\bar{x}$	0.52	0.92	0.39
	sd	0.05	0.1	0.05
40.0	$\bar{x}$	0.52	0.93	0.38
	sd	0.07	0.5	0.02
80.0	$\bar{x}$	0.52	0.92	0.39
	sd	0.04	0.05	0.06
140.0	$\bar{x}$	0.53	0.93	0.38
	sd	0.01	0.15	0.14

Fig. 3.6 Influence of K on yield in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 µmol $m^{-2}s^{-1}$

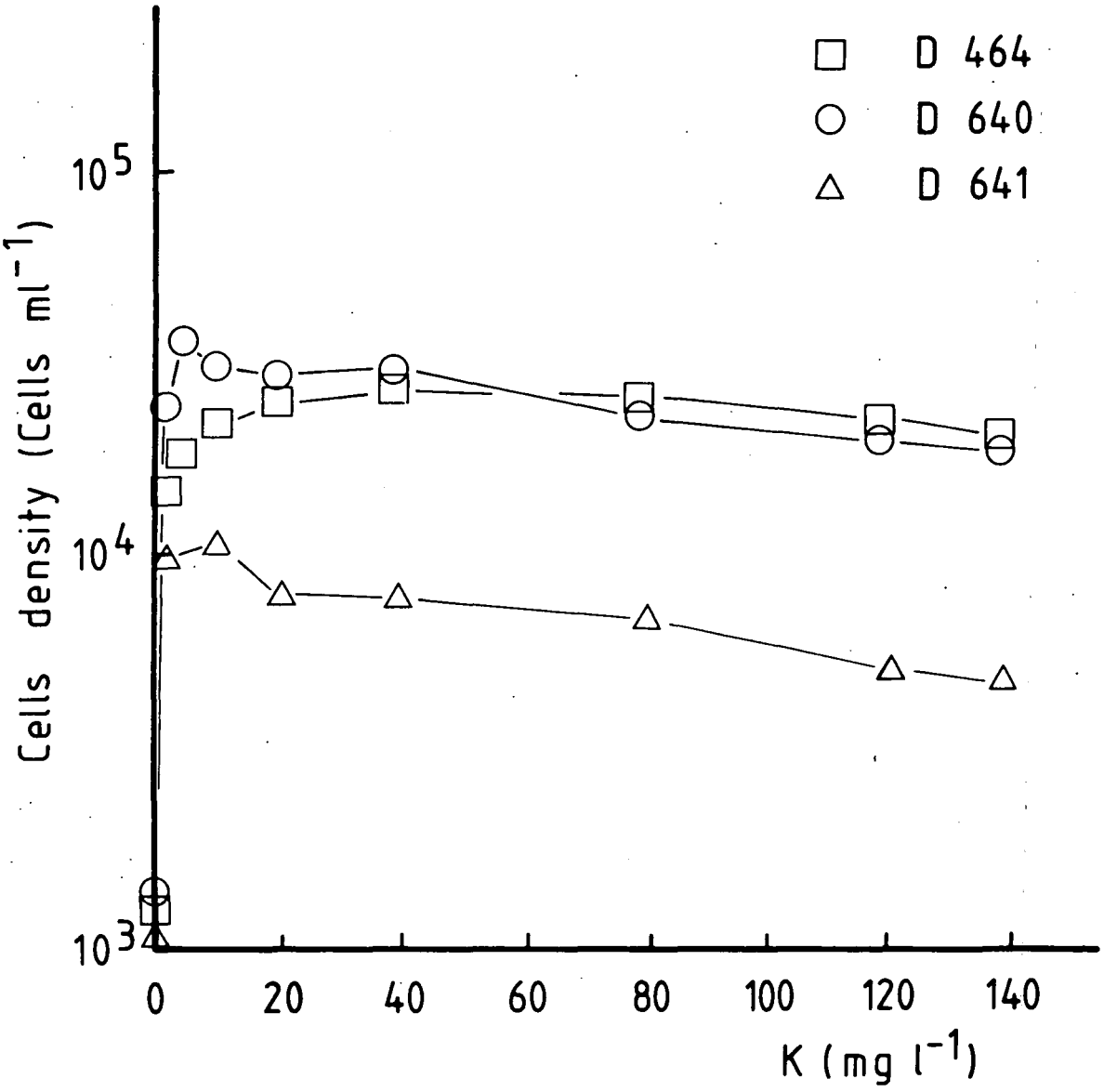

D 464
D 640
D 641
10^5
10^4
10^3
Cells density (Cells ml^{-1})
0
20
40
60
80
100
120
140
K (mg l^{-1})

Fig. 3.7 Influence of Mg on yield in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 µmol $m^{-2}s^{-1}$

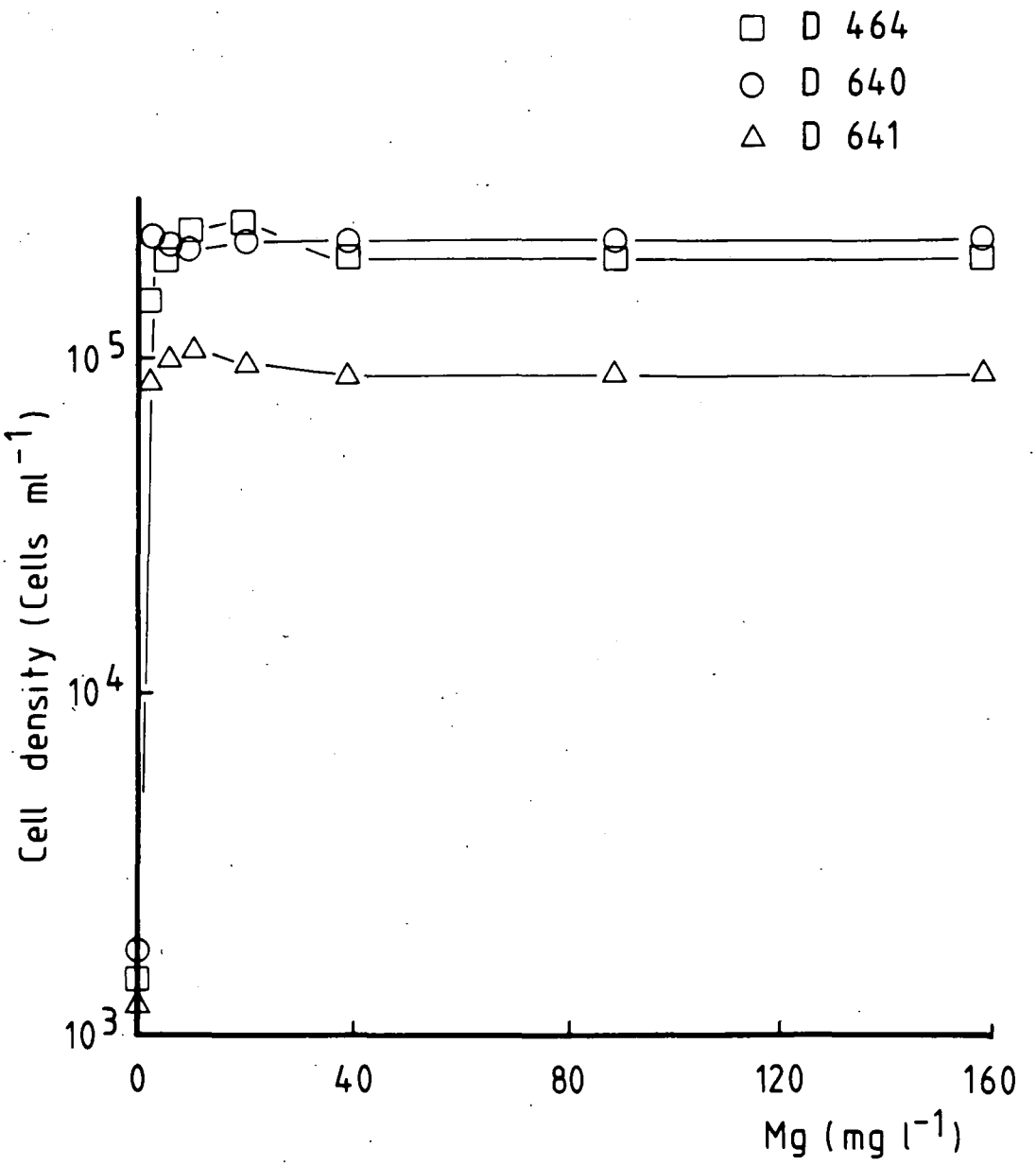

D 464
D 640
D 641
Cell density (Cells ml^{-1})
10^5
10^4
10^3
0
40
80
120
160
Mg (mg l^{-1})

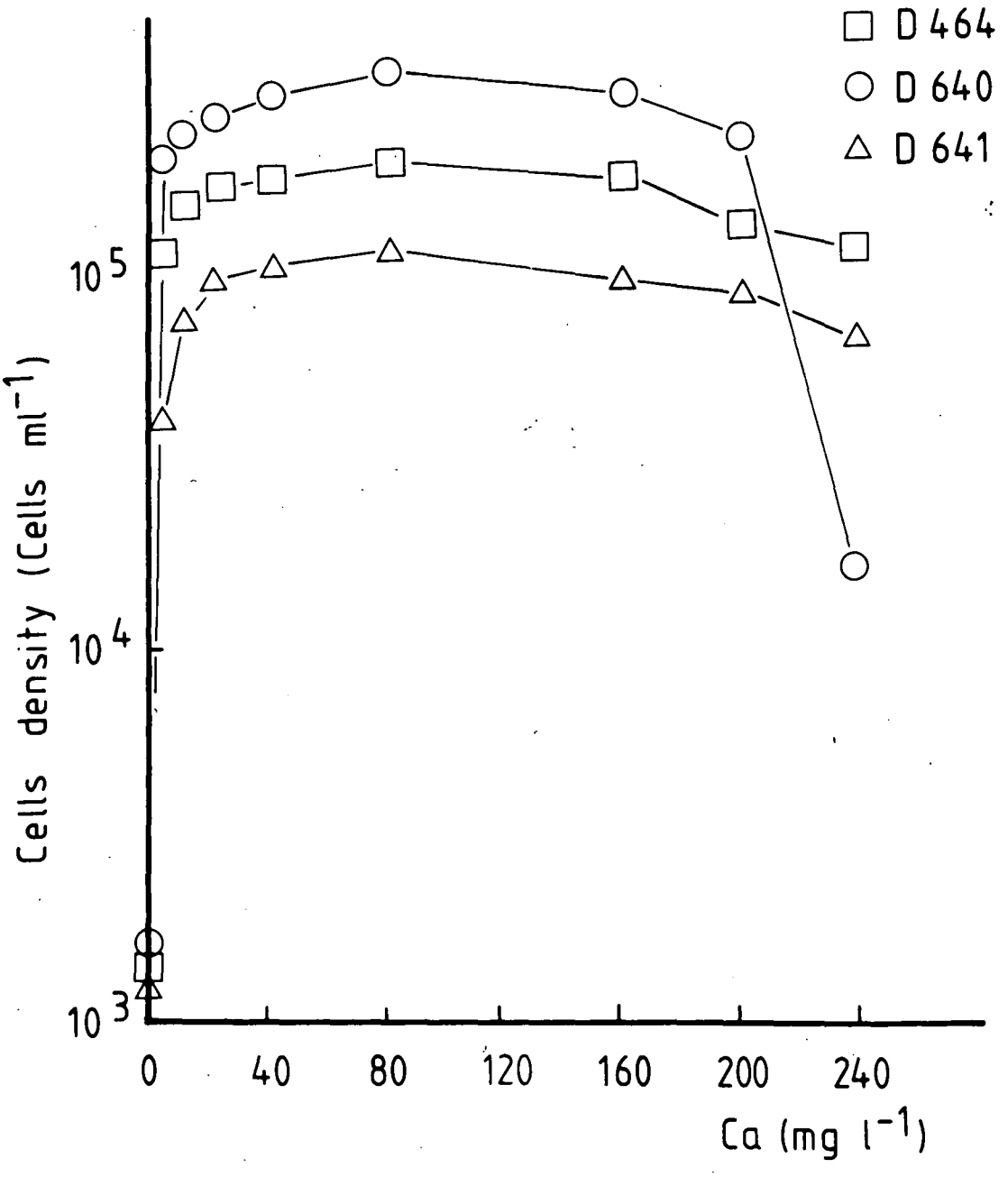
TABLE 3.10 Influence of magnesium on yield in *E. mutabilis* D464, D640 and D641 measured as dry weight ($g\ l^{-1}$) in batch culture in 20 days at 25°C and photon flux density of 100 $\mu mol\ m^{-2}\ s^{-1}$; n = 16.

Mg ($mg\ l^{-1}$)		D464	D640	D641
2.5	$\bar{x}$	0.52	0.91	0.42
	sd	0.01	0.06	0.02
5.0	$\bar{x}$	0.69	0.89	0.56
	sd	0.05	0.01	0.06
10.0	$\bar{x}$	0.87	0.87	0.55
	sd	0.01	0.05	0.02
20.0	$\bar{x}$	0.87	0.87	0.55
	sd	0.1	0.01	0.05
40.0	$\bar{x}$	0.86	0.88	0.55
	sd	0.01	0.04	0.02
80.0	$\bar{x}$	0.87	0.89	0.54
	sd	0.1	0.05	0.04
160.0	$\bar{x}$	0.86	0.88	0.54
	sd	0.04	0.02	0.02

TABLE 3.11 Influence of calcium on yield (dry weight in ($g\ l^{-1}$) in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 $\mu mol\ m^{-2}\ s^{-1}$; n = 16.

Ca ($mg\ l^{-1}$)		D464	D640	D641
5.0	$\bar{x}$	0.42	0.73	0.24
	sd	0.01	0.04	0.03
10.0	$\bar{x}$	0.52	0.92	0.43
	sd	0.04	0.02	0.04
20.0	$\bar{x}$	0.74	0.97	0.51
	sd	0.1	0.04	0.04
40.0	$\bar{x}$	0.97	1.12	0.69
	sd	0.04	0.05	0.04
80.0	$\bar{x}$	1.03	1.24	0.73
	sd	0.4	0.3	0.04
160.0	$\bar{x}$	0.99	1.03	0.57
	sd	0.3	0.04	0.04
200.0	$\bar{x}$	0.79	0.97	0.54
	sd	0.1	0.04	0.2
240.0	$\bar{x}$	0.52	0.31	0.51
	sd	0.04	0.01	0.04

Fig. 3.8 Influence of Ca on yield in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 µmol $m^{-2}s^{-1}$


D 464
D 640
D 641
Cells density (Cells ml^{-1})
10^5
10^4
10^3
0
40
80
120
160
200
240
Ca (mg l^{-1})

absence of the particular element under investigation, experiments were conducted with different concentrations of microelements. Each experiment was carried out four times with four replicates for each experimental condition.

The basal medium had the following concentrations of metals:

Zn 0.013 mg l^{-1}

Cu 0.002 mg l^{-1}

Co 0.002 mg l^{-1}

Mn 0.01 mg l^{-1}

3.52 Influence of zinc on yield

Effect of Zn on yield in *Euglena mutabilis* D464, D640 and D641 was measured after 20 days of incubation in the basal medium with different concentrations of Zn. The results (Fig.3.9) indicated improved yield with Zn (Table 3.12).

3.53 Influence of copper on yield

The results (Fig.3.10) of the experiments conducted to see the influence of Cu on growth in *Euglena mutabilis* indicated an optimum yield at 0.005 mg l^{-1} Cu in strain D464 and D641 while strain D640 was at 0.002 mg l^{-1}. Further increase reduced yield (Table 3.13).

3.54 Influence of manganese on yield

The influence of Mn on yield in *Euglena mutabilis* strain D464, D640 and D641 was checked with different concentrations of Mn in the basal medium at pH 2.6. Results

TABLE 3.12 Influence of zinc on yield measured as dry weight (g l^{-1}) in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}; n = 16.

Zn (mg l^{-1})		D464	D640	D641
-Zn	$\bar{x}$	0.41	0.54	0.21
	sd	0.01	0.03	0.05
0.013 (basal medium)	$\bar{x}$	0.51	0.92	0.43
	sd	0.04	0.03	0.01
0.05	$\bar{x}$	0.74	0.94	0.52
	sd	0.02	0.04	0.01
0.5	$\bar{x}$	0.97	1.03	0.63
	sd	0.1	0.13	0.04
1.0	$\bar{x}$	1.23	0.97	0.61
	sd	0.14	0.04	0.03
2.0	$\bar{x}$	1.13	0.94	0.57
	sd	0.5	0.03	0.02
4.0	$\bar{x}$	1.03	0.89	0.54
	sd	0.2	0.04	0.02
6.0	$\bar{x}$	0.97	0.87	0.55
	sd	0.04	0.03	0.01
8.0	$\bar{x}$	0.83	0.84	0.47
	sd	0.04	0.01	0.01
10.0	$\bar{x}$	0.63	0.84	0.40
	sd	0.01	0.04	0.02

Fig. 3.9 Influence of Zn on yield in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25^{o}C and photon flux density of 100 µmol $m^{-2}s^{-1}$

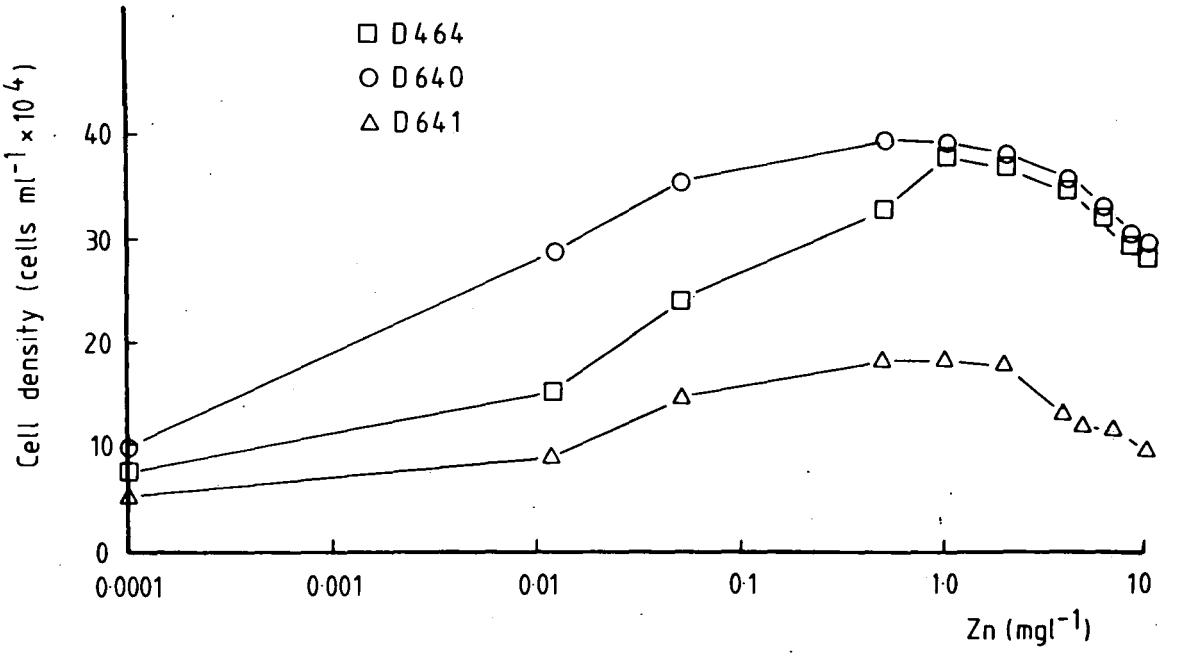

□ D464
○ D640
△ D641
Cell density (cells ml^{-1} x 10^4)
40
30
20
10
0
0·0001
0·001
0·01
0·1
1·0
10
Zn (mgl^{-1})

TABLE 3.13 Influence of copper on yield as dry weight ($g\ l^{-1}$) in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 $\mu mol\ m^{-2}\ s^{-1}$; n = 16.

Cu ($mg\ l^{-1}$)		D464	D640	D641
-Cu	$\bar{x}$	0.40	0.41	0.29
	sd	0.03	0.03	0.01
0.002	$\bar{x}$	0.44	0.45	0.35
	sd	0.01	0.04	0.02
0.005 (basal medium)	$\bar{x}$	0.51	0.91	0.42
	sd	0.03	0.01	0.02
0.01	$\bar{x}$	0.49	0.83	0.41
	sd	0.02	0.05	0.01
0.05	$\bar{x}$	0.47	0.81	0.39
	sd	0.1	0.04	0.04
0.1	$\bar{x}$	0.44	0.77	0.37
	sd	0.01	0.03	0.05
0.2	$\bar{x}$	0.41	0.69	0.35
	sd	0.02	0.04	0.01
0.4	$\bar{x}$	0.40	0.58	0.35
	sd	0.04	0.03	0.01
0.6	$\bar{x}$	0.37	0.56	0.34
	sd	0.04	0.01	0.02
0.8	$\bar{x}$	0.38	0.51	0.31
	sd	0.03	0.01	0.02
1.0	$\bar{x}$	0.34	0.47	0.30
	sd	0.01	0.03	0.02

Fig. 3.10 Influence of Cu on yield in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 µmol $m^{-2}s^{-1}$

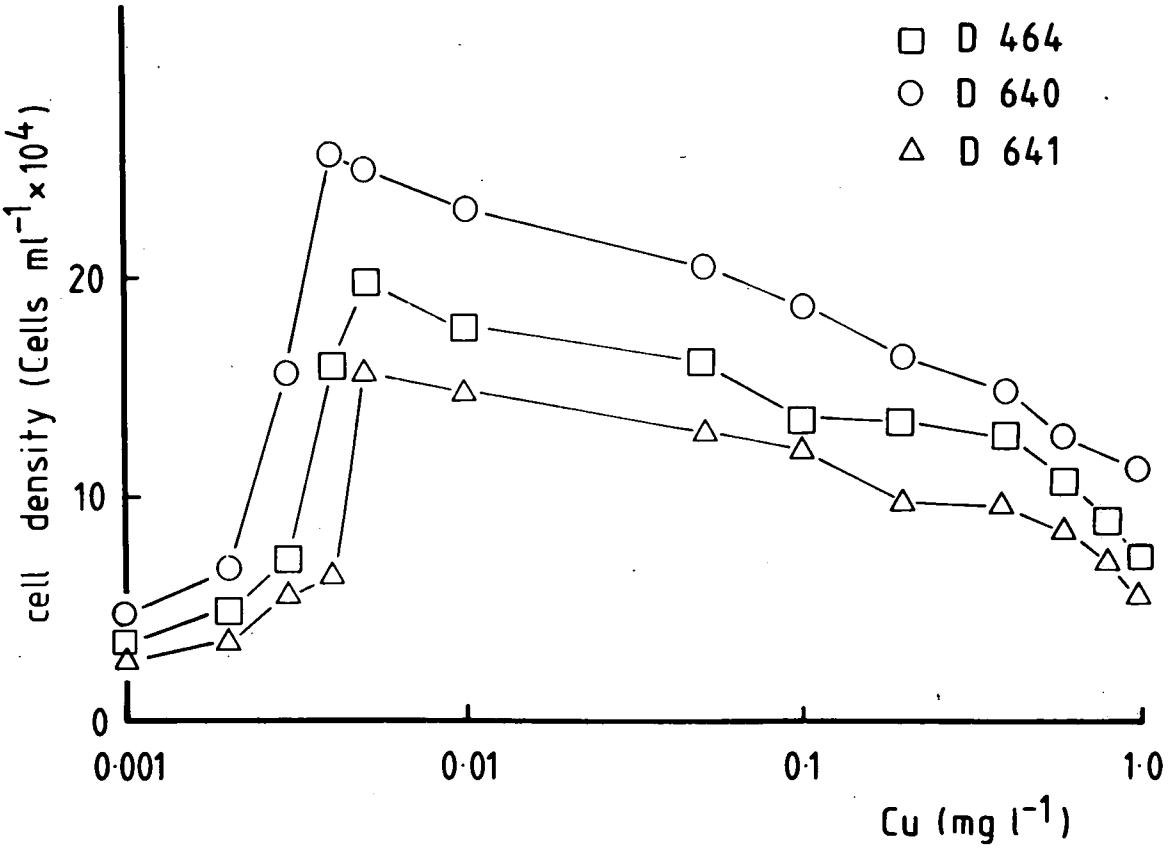
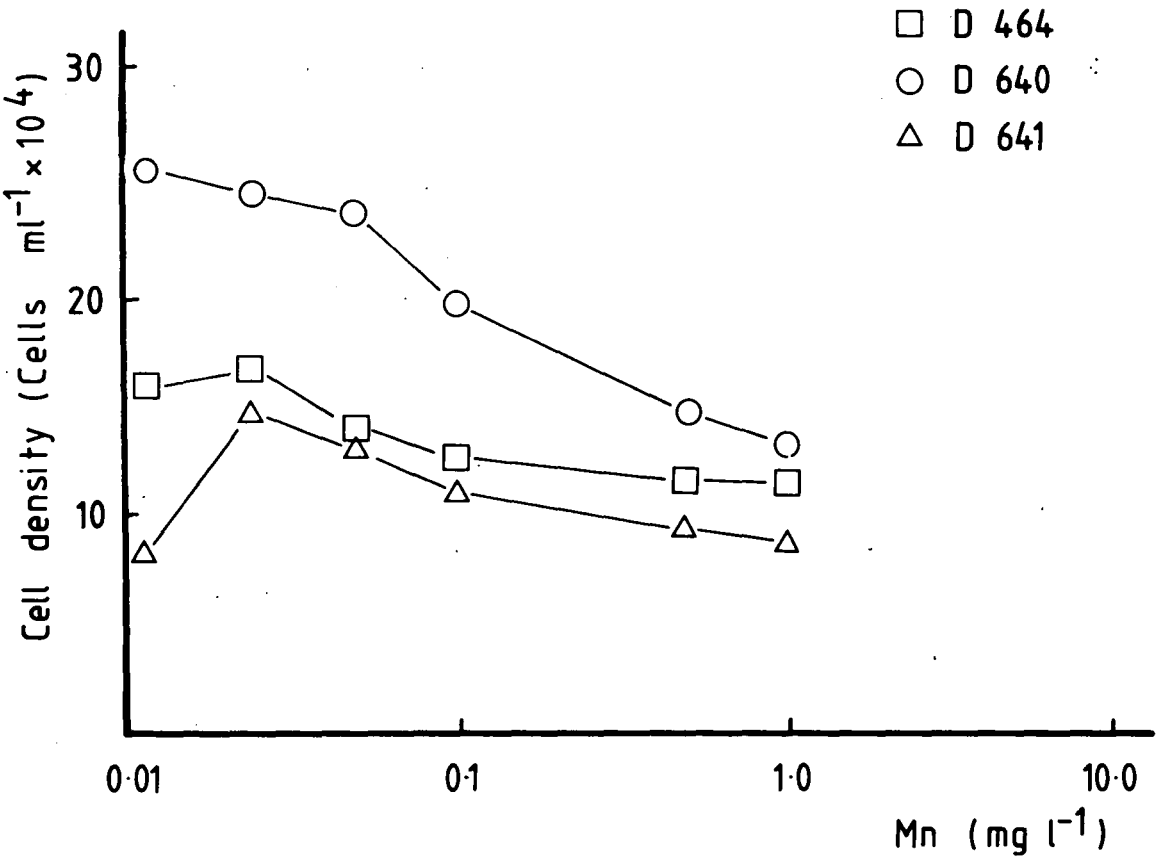

D 464
D 640
D 641
cell density (Cells ml^{-1} x 10^4)
20
10
0
0·001
0·01
0·1
1·0
Cu (mg l^{-1})

Fig. 3.11 Influence of Mn on yield in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}


D 464
D 640
D 641
Cell density (Cells ml^{-1} x 10^4)
30
20
10
0·01
0·1
1·0
10·0
Mn (mg l^{-1})

(Fig.3.11) indicated an optimum yield at 0.025 mg l^{-1} for strain D464 and D641 and D640 was at 0.01 mg l^{-1}. Increased concentration above 0.025 brought about a reduction in the yield (Table 3.14).

3.55 Influence of cobalt on yield

With inoculum from Co-free medium, *Euglena mutabilis* D464, D640 and D641 showed improved growth in the 0.002 mg l^{-1} Co. Further increase from 0.002 to 0.05 showed no adverse effect on yield. With increased Co concentration from 0.05 to 1.0 mg l^{-1} there was a reduction in the yield (Fig.3.12; Table 3.15).

3.6 Photoheterotrophic and heterotrophic growth

3.61 Introduction

Preliminary investigation indicated that *Euglena mutabilis* D464, D640 and D641 can utilise a number of organic nitrogen and phosphorus in the growth media at pH 2.6 and also carbon substrates in the presence of atmospheric carbon (CO_2). Experiments were set up to compare growth in the different organic substrates (Table 2.4). Parallel experiments were conducted in the basal growth medium as control. Using standard inocula of 10^2 cell ml^{-1} from cultures grown in organic substrates, growth as yield was checked at 25°C and photon flux density of 100 µmol m^{-2} s^{-1} in 20 days in different organic substrates. Each experiment was repeated four times with four replicates.

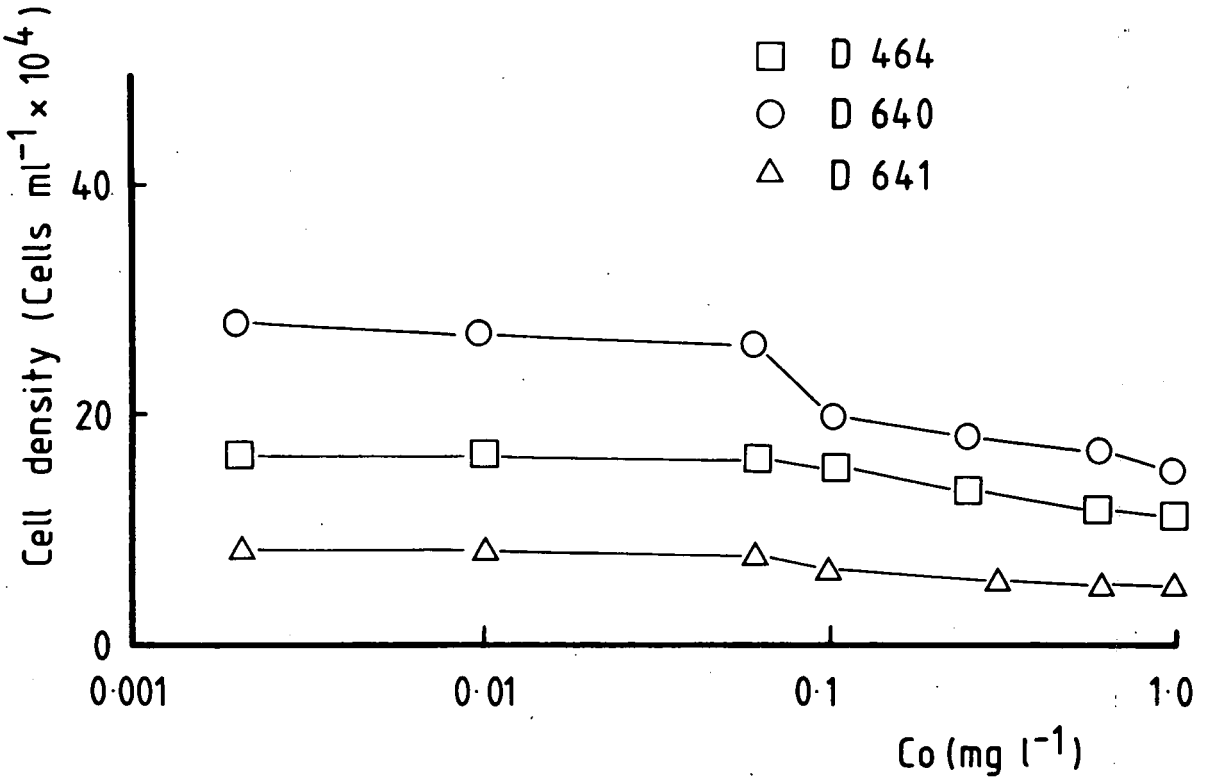
TABLE 3.14 Influence of manganese on yield measured as dry weight ($g\ l^{-1}$) in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 $\mu mol\ m^{-2}\ s^{-1}$; n = 16.

Mn ($mg\ l^{-1}$)		D464	D640	D641
0	$\bar{x}$	0.41	0.54	0.37
	sd	0.04	0.01	0.03
0.012 (basal medium)	$\bar{x}$	0.52	0.91	0.42
	sd	0.01	0.03	0.01
0.025	$\bar{x}$	0.54	0.87	0.51
	sd	0.03	0.02	0.04
0.05	$\bar{x}$	0.50	0.84	0.51
	sd	0.02	0.03	0.01
0.1	$\bar{x}$	0.47	0.77	0.47
	sd	0.04	0.03	0.01
0.5	$\bar{x}$	0.47	0.61	0.41
	sd	0.02	0.01	0.03
1.0	$\bar{x}$	0.41	0.51	0.39
	sd	0.03	0.04	0.01

TABLE 3.15 Influence of cobalt on yield measured as dry weight (g l^{-1}) in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}; n = 16.

Co (mg l^{-1})		D464	D640	D641
0	$\bar{x}$	0.41	0.57	0.30
	sd	0.01	0.03	0.04
0.002 (basal medium)	$\bar{x}$	0.52	0.92	0.42
	sd	0.02	0.04	0.01
0.01	$\bar{x}$	0.52	0.91	0.40
	sd	0.02	0.01	0.03
0.05	$\bar{x}$	0.52	0.87	0.37
	sd	0.01	0.04	0.03
0.1	$\bar{x}$	0.51	0.76	0.34
	sd	0.04	0.03	0.03
0.25	$\bar{x}$	0.49	0.72	0.31
	sd	0.01	0.04	0.02
0.5	$\bar{x}$	0.44	0.69	0.31
	sd	0.04	0.03	0.02
1.0	$\bar{x}$	0.42	0.52	0.32
	sd	0.01	0.02	0.01

Fig. 3.12 Influence of Co on yield in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 µmol $m^{-2}s^{-1}$


D 464
D 640
D 641
Cell density (Cells ml^{-1} x 10^{4})
40
20
0
0·001
0·01
0·1
1·0
Co (mg l^{-1})

3.62 Influence of organic nitrogen on yield

The influence of alanine, asparagine, glycyl-glycine, glycine,urea, uric acid and ethanolamine (Table 2.2) on yield in *Euglena mutabilis* was checked after 20 days of incubation under standard growth conditions. Results (Fig.3.13) indicate better growth (yield) in glycyl-glycine and asparagine compared with yield in the basal medium (Table 3.16).

TABLE 3.16 Influence of organic nitrogen on yield measured as dry weight (g l^{-1}) in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}; n=16.

		D464	D640	D641
Basal medium	$\bar{x}$	0.52	0.92	0.41
	sd	0.02	0.04	0.02
Alanine	$\bar{x}$	0.40	0.99	0.31
	sd	0.03	0.04	0.01
Asparagine	$\bar{x}$	0.73	1.07	0.44
	sd	0.04	0.1	0.03
Glycine	$\bar{x}$	0.51	0.71	0.43
	sd	0.03	0.01	0.04
Glycyl-glycine	$\bar{x}$	1.42	1.14	1.17
	sd	0.2	0.3	0.1
Urea	$\bar{x}$	0.31	0.33	0.72
	sd	0.04	0.03	0.03
Uric acid	$\bar{x}$	0.34	0.37	0.74
	sd	0.04	0.01	0.03
Ethanolamine	$\bar{x}$	0.33	0.33	0.47
	sd	0.02	0.01	0.04

3.63 Influence of organic phosphate on yield

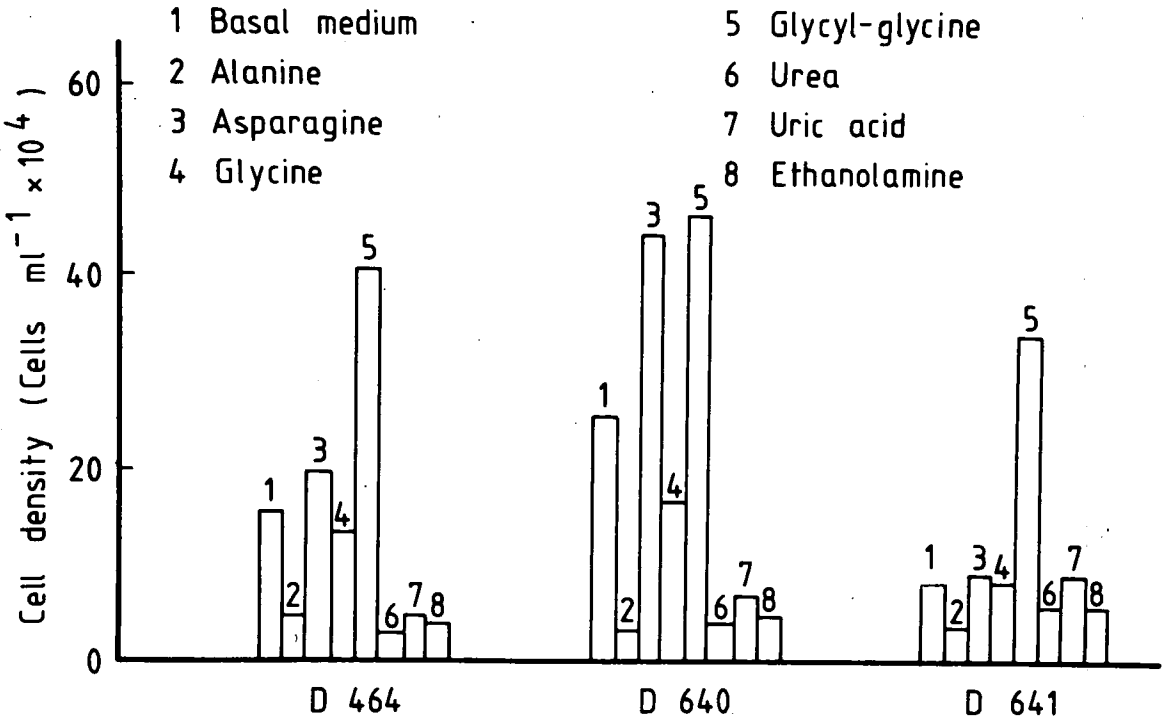
With β-glycerophosphate, DNA and phytic acid whose stability over the usual incubation period of 20 days was seen to be stable (Section 2.45), yield was checked and was found to be similar to that of the basal medium in D641, whereas in D464, and D640, yield was better in the basal medium (Fig. 3.14, Table 3.17).

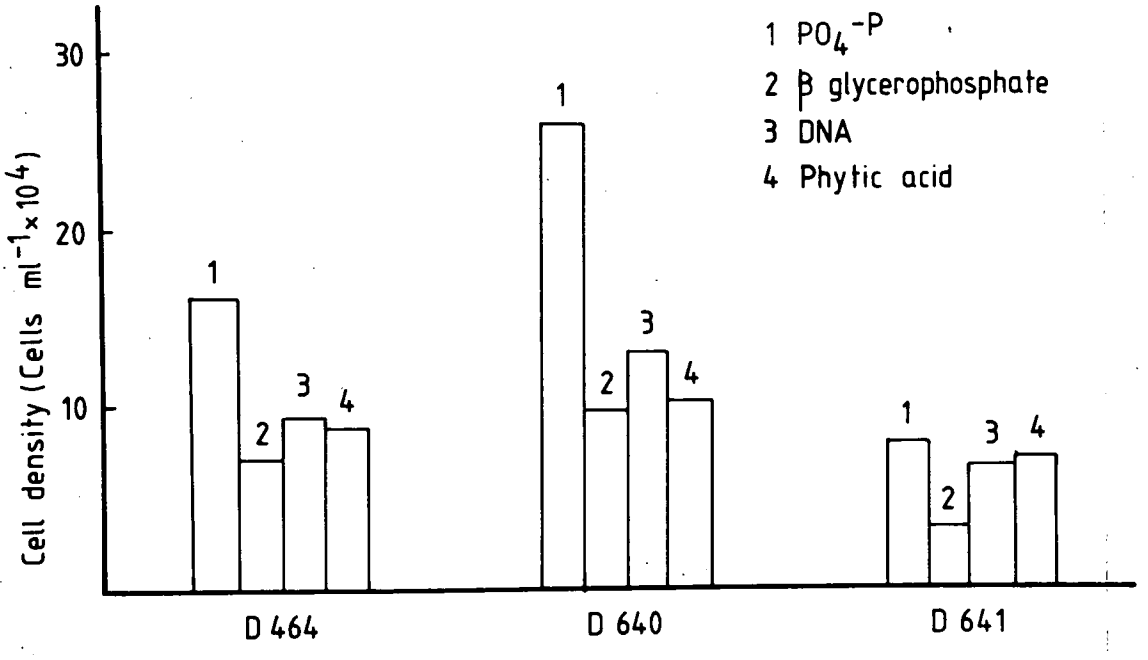
TABLE 3.17 Influence of organic phosphate on yield measured as dry weight (g l^{-1}) in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}; n=16.

		D464	D640	D641
Basal medium	$\bar{x}$	0.52	0.91	0.42
	sd	0.02	0.04	0.01
β-glycerophosphate	$\bar{x}$	0.39	0.52	0.37
	sd	0.03	0.02	0.01
DNA	$\bar{x}$	0.41	0.63	0.41
	sd	0.04	0.02	0.01
Phytic acid	$\bar{x}$	0.40	0.53	0.42
	sd	0.04	0.02	0.03

Fig. 3.13 Influence of organic nitrogen on yield in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 μmol m^{-2} s^{-1}

Fig. 3.14 Influence of organic phosphate on yield in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 μmol m^{-2} s^{-1}


1 Basal medium
2 Alanine
3 Asparagine
4 Glycine
5 Glycyl-glycine
6 Urea
7 Uric acid
8 Ethanolamine
Cell density (Cells ml⁻¹ x 10⁴)
60
40
20
0
D 464
D 640
D 641


1 PO4-P
2 β glycerophosphate
3 DNA
4 Phytic acid
Cell density (Cells ml⁻¹ x 10⁴)
30
20
10
D 464
D 640
D 641

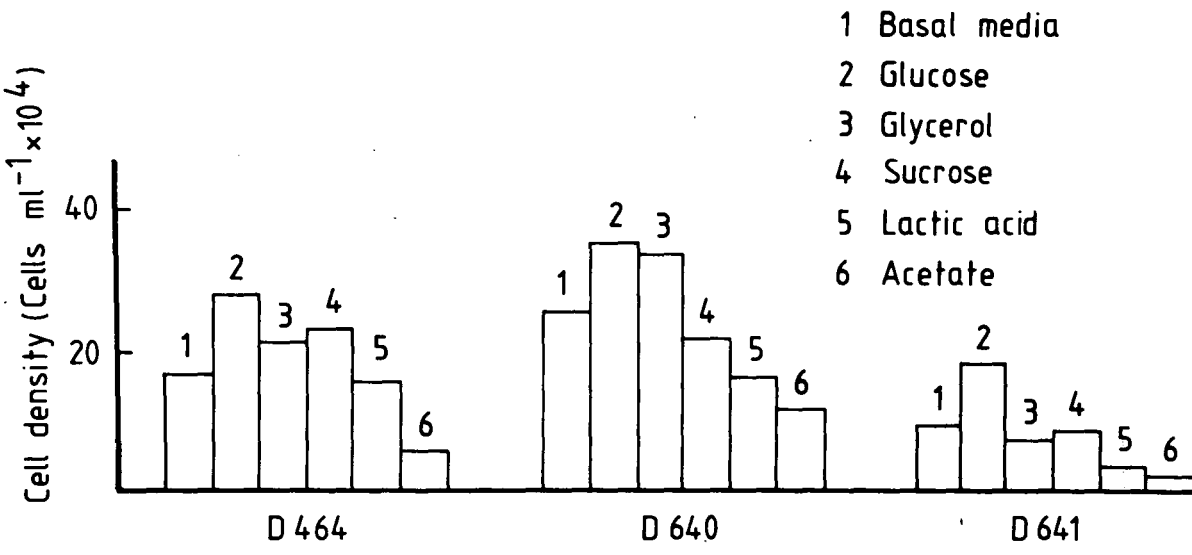
3.64 Influence of organic carbon on yield

With organic carbon (Table 2.2) all three strains had improved growth (yield) in glucose. The lowest yield was in acetate (Fig. 3.16, Table 3.18).

TABLE 3.18 Influence of organic carbon on yield measured as dry weight ($g\ l^{-1}$) in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 $\mu mol\ m^{-2}\ s^{-1}$; n=16.

		D464	D640	D641
Basal medium	$\bar{x}$	0.51	0.91	0.41
	sd	0.03	0.04	0.02
Glucose	$\bar{x}$	0.92	1.32	0.52
	sd	0.04	0.1	0.03
Glycerol	$\bar{x}$	0.79	0.97	0.39
	sd	0.04	0.04	0.01
Sucrose	$\bar{x}$	0.87	0.72	0.40
	sd	0.03	0.04	0.01
Lactic acid	$\bar{x}$	0.63	0.47	0.37
	sd	0.02	0.04	0.01
Acetate	$\bar{x}$	0.42	0.39	0.27
	sd	0.04	0.01	0.03

Fig. 3.15 Influence of organic carbon on yield in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25^{o}C and photon flux density of 100 µmol m^{-2} s^{-1}


1 Basal media
2 Glucose
3 Glycerol
4 Sucrose
5 Lactic acid
6 Acetate
Cell density (Cells ml^{-1} x 10^4)
40
20
1
2
3
4
5
6
D 464
D 640
D 641

3.65 Influence of vitamins on yield

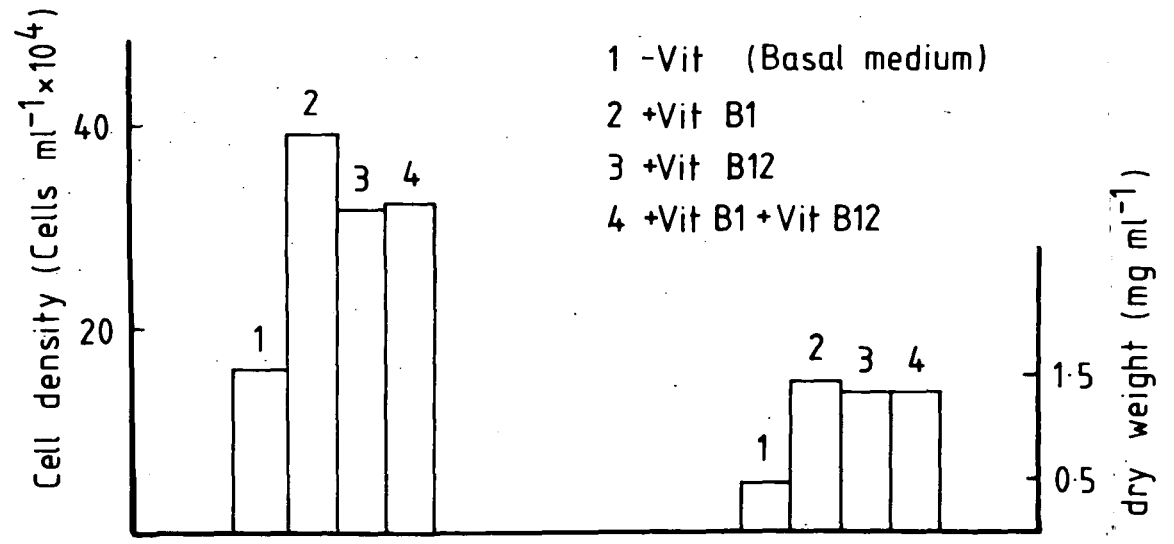
Experiments conducted with *Euglena mutabilis* D464 in the presence of 1 µg l^{-1} vitamin B_1 and 1 mg l^{-1} vitamin B_{12}, both singly and in combination in the basal medium, indicated better yield in the presence of added vitamins (Figs. 3.16 and 16a). Experiments conducted replacing Co with vitamin B_{12} too improved yield (Table 3.19).

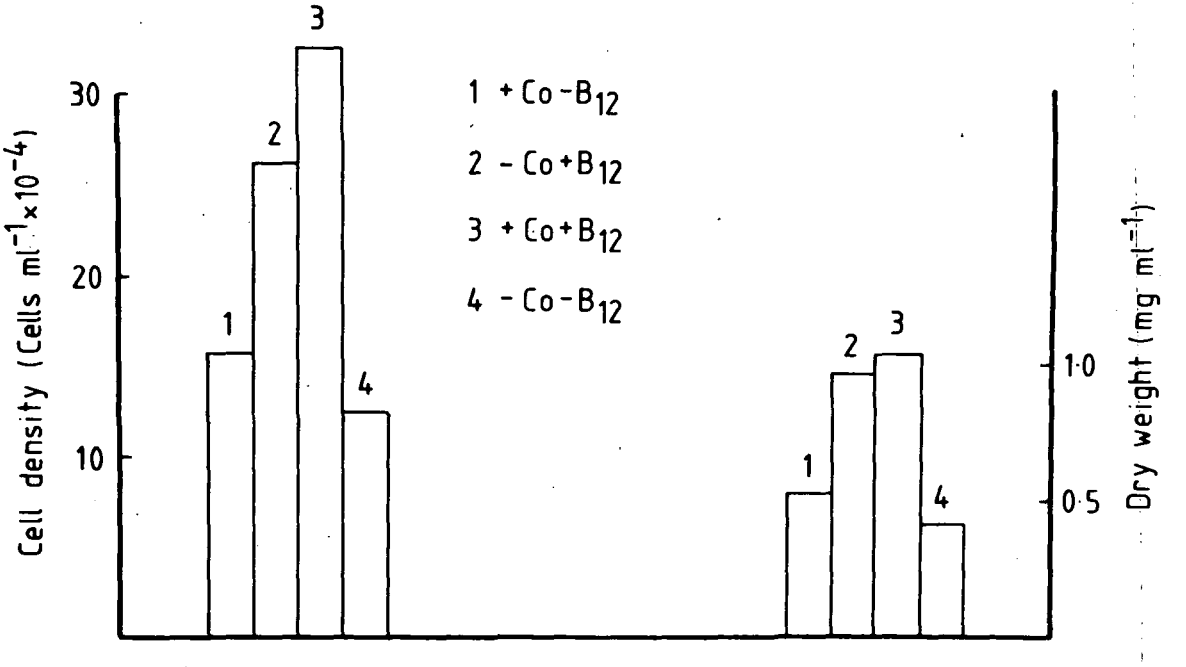
TABLE 3.19 Influence of vitamins on yield measured as dry weight in *E. mutabilis* D464 in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}; n = 16.

Basal medium	$\bar{x}$	0.51	g l^{-1}
(vitamin free)	sd	0.03	
+ vitamin B_1	$\bar{x}$	1.28	g l^{-1}
(1 µg l^{-1})	sd	0.1	
+ vitamin B_{12}	$\bar{x}$	1.11	g l^{-1}
(1 µg l^{-1})	sd	0.2	
+ vitamin B_1 and	$\bar{x}$	1.03	g l^{-1}
vitamin B_{12}	sd	0.1	

Fig. 3.16 Influence of vitamin B_1 and B_{12} on yield in *E. mutabilis* D464 in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}

Fig. 3.16a Influence of Co and B_{12} on yield in *E. mutabilis* D464 in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}


1 -Vit (Basal medium)
2 +Vit B1
3 +Vit B12
4 +Vit B1 + Vit B12
Cell density (Cells ml^{-1} x 10^4)
40
20
1
2
3
4
1·5
0·5
dry weight (mg ml^{-1})


1 + Co-B$_{12}$
2 - Co+B$_{12}$
3 + Co+B$_{12}$
4 - Co-B$_{12}$
Cell density (Cells ml^{-1} x 10^{-4})
30
20
10
1·0
0·5
Dry weight (mg ml^{-1})

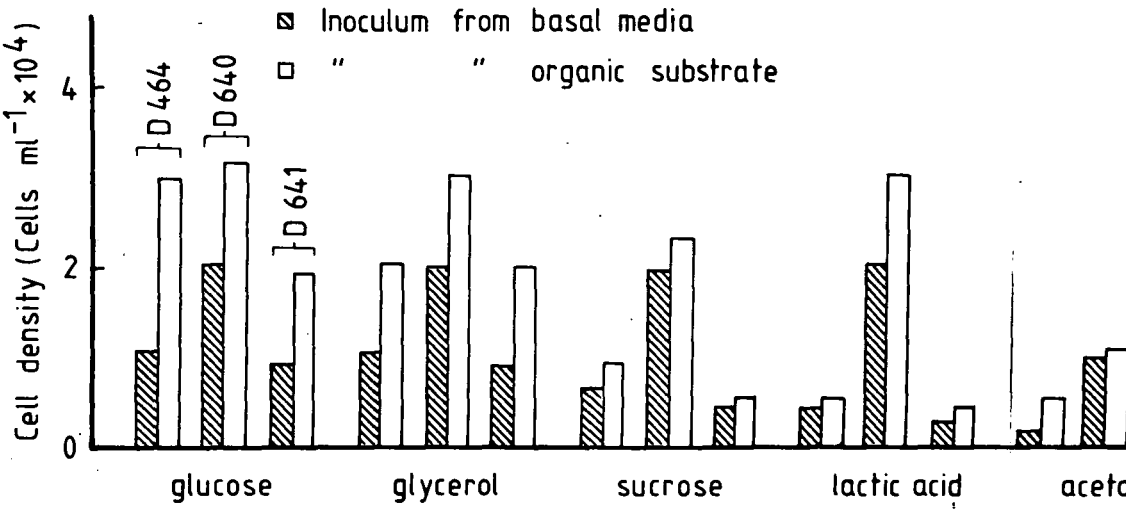
3.66 Utilization of organic carbon and nitrogen in the dark

In order to check whether *Euglena mutabilis* could utilize organic carbon and nitrogen substrates in the absence of light, strain D464, D640 and D641 were subcultured using inocula from stocks grown in the basal medium and from stocks grown in organic substrates under investigation (Section 2.42). Results (Fig.3.17) indicated that they could utilize different organic carbon and nitrogen substrates. *Euglena mutabilis* D640 (estuarine origin) had better yield in all substrates including acetate in which substrate D464 and D641 had very low yield (Table 3.20). There was no growth in any of the nitrogen substrates tested.

TABLE 3.20 Influence of organic carbon substrates on dark heterotrophic growth in *E. mutabilis* D464, D640 and D641 measured as dry weight ($g\ l^{-1}$) in 60 days at 25°C. Inoculum from organic substrates

		D640	D464	D641
Glucose	$\bar{x}$	0.47	0.42	0.34
	sd	0.04	0.01	0.03
Glycerol	$\bar{x}$	0.44	0.33	0.32
	sd	0.01	0.04	0.03
Sucrose	$\bar{x}$	0.41	0.24	0.31
	sd	0.02	0.04	0.01
Lactic acid	$\bar{x}$	0.44	0.21	0.32
	sd	0.03	0.03	0.01
Acetate (Na salt)	$\bar{x}$	0.25	0.23	0.19
	sd	0.03	0.01	0.04

Fig. 3.17 Influence of organic carbon substrates on yield in dark heterotrophic growth in *E. mutabilis* D464 in 20 days at 25°C and photon flux density of 100 μmol m^{-2} s^{-1}


Inoculum from basal media
" " organic substrate
Cell density (Cells ml^{-1} x 10^4)
0
2
4
D 464
D 640
D 641
glucose
glycerol
sucrose
lactic acid

3.67 Morphological changes in heterotrophic growth

Cells examined after 5 days of incubation in organic substrates, many of the cells in lactic acid, acetate, alanine, urea, uric acid and ethanolamine were rounded up (*c*.26 µm in dia.) but green. In substrates in which growth occurred, cells were often seen stuck in a clear transparent jelly-like substance. In dark grown cultures cells without chloroplast and eye spots were a common feature. In both light and dark grown organic carbon substrates cells were seen in the mist of globular bodies of *c*. 7.9 to 11.8 µm dia. With organic nitrogen substrates cells grown in glycyl-glycine had similar bodies but were smaller (*c*. 3.8 to 4.5 µm dia.).

3.7 Heavy metals tolerance in *Euglena mutabilis*

3.71 Introduction

The influence of a number of heavy metals, Zn, Cu, Fe, Al, Pb, Ni, Co and Cd was examined using three strains of *Euglena mutabilis*. Experiments were conducted static using 20 ml of culture media at pH 2.6 at a photon flux density of 100 mol m^{-2} s^{-1} in the growth room at 25°C. Each experiment was carried out four times with four replicates. Cell count and dry weight measurements were done on the 20th day after inoculation.

The basal medium has the following concentration of metals:

Zn	0.013	mg l^{-1}
Cu	0.004	"
Mn	0.012	"
Fe	0.5	"
Al	0.0	"
Pb	0.0001	"
Cd	0.0001	"
Ni	0.0001	"
Co	0.002	"

3.72 Influence of zinc on yield

E. mutabilis D464, D640 and D641 were subjected to different concentrations of Zn in the basal medium. Growth measured as yield in 20 days indicated that all three strains tolerate similar Zn concentration though they were isolated from different field Zn concentration and all three strains recovered from exposure to 80 mg l^{-1} Zn (Fig.3.18 and Table 3.21).

3.73 Influence of copper on yield

Response to Cu concentration up to 1 mg l^{-1} was similar in all three strains of *E. mutabilis*. Further increases up to 75 mg l^{-1} indicated tolerance up to 40 mg l^{-1} Cu with higher tolerance in D641, (Fig. 3.19 and Table 3.22). There was no recovery from exposure to a concentration of 75 mg l^{-1} Cu in 20 days.

TABLE 3.21 Influence of zinc on yield measured as dry weight ($g\ l^{-1}$) in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 $\mu mol\ m^{-2}\ s^{-1}$ n = 16.

		D464	D640	D641
$ZnSO_4$ ($mg\ l^{-1}$)				
0.013 (basal medium	$\bar{x}$	0.51	0.92	0.41
	sd	0.04	0.02	0.01
1.0	$\bar{x}$	1.23	0.97	0.61
	sd	0.14	0.04	0.03
5.0	$\bar{x}$	1.03	0.87	0.52
	sd	0.04	0.02	0.01
10.0	$\bar{x}$	0.63	0.84	0.40
	sd	0.01	0.02	0.04
20.0	$\bar{x}$	0.42	0.41	0.31
	sd	0.02	0.01	0.04
40.0	$\bar{x}$	0.17	0.23	0.12
	sd	0.03	0.04	0.01
$ZnCl_2$ ($mg\ l^{-1}$)				
1.0	$\bar{x}$	1.24	1.11	0.76
	sd	0.11	0.1	0.04
5.0	$\bar{x}$	1.10	0.89	0.54
	sd	0.1	0.04	0.01
10.0	$\bar{x}$	0.60	0.82	0.40
	sd	0.03	0.01	0.04
20.0	$\bar{x}$	0.42	0.41	0.32
	sd	0.02	0.01	0.03
40.0	$\bar{x}$	0.18	0.25	0.15
	sd	0.01	0.04	0.01
50.0	$\bar{x}$	0.15	0.20	0.15
	sd	0.01	0.04	0.02

Fig. 3.18 Influence of Zn on yield in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 μmol m^{-2} s^{-1}

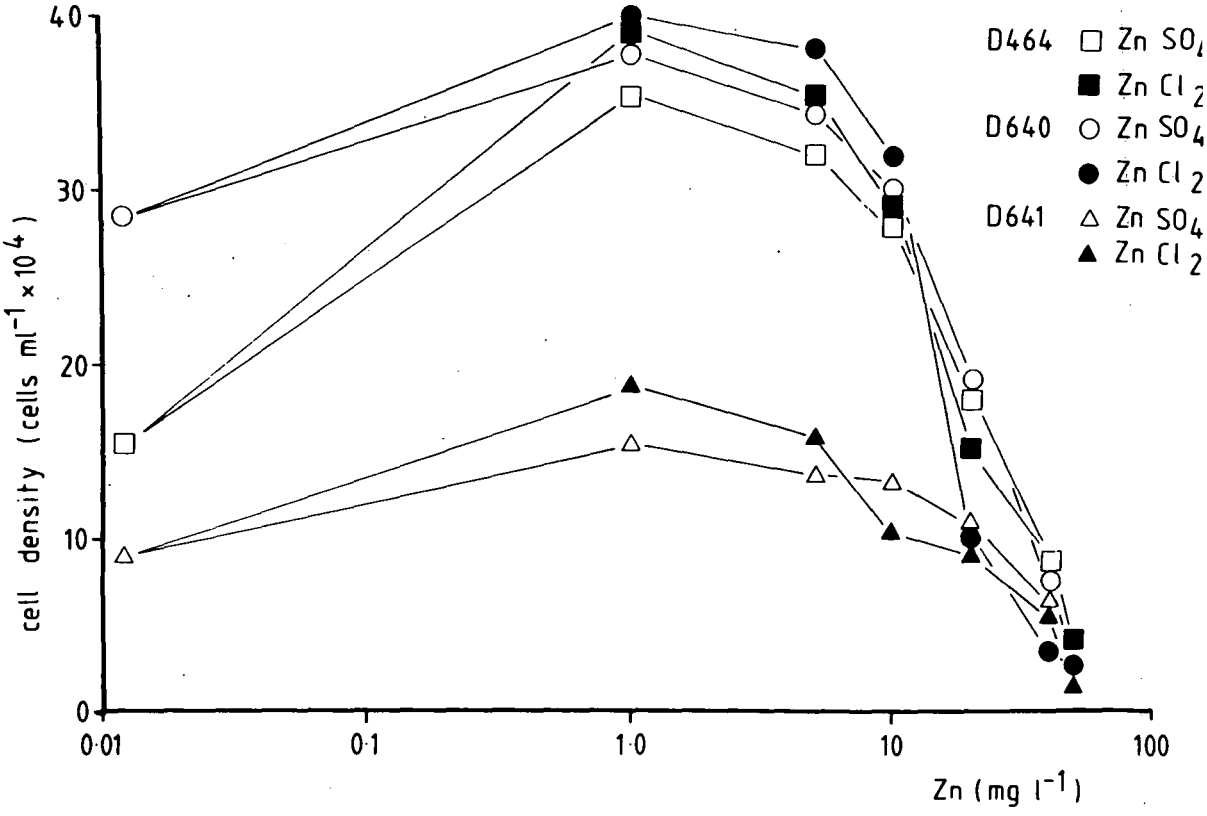
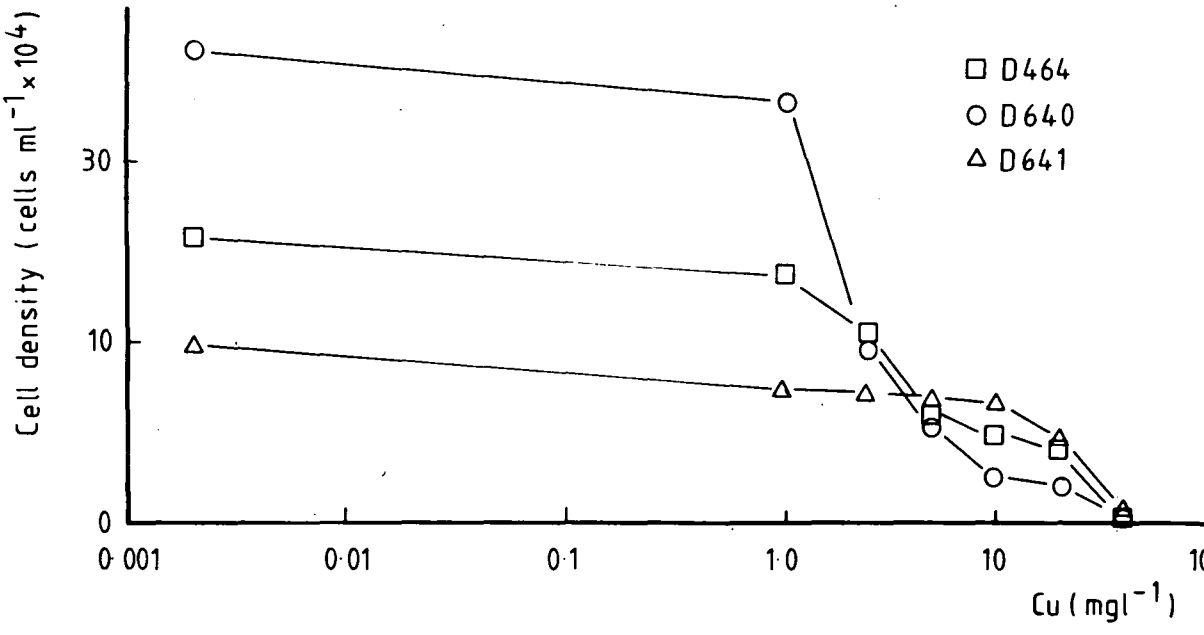

40
30
20
10
0
cell density (cells ml^{-1} x 10^{-4})
0·01
0·1
1·0
10
100
Zn (mg l^{-1})
D464
Zn SO$_4$
Zn Cl$_2$
D640
Zn SO$_4$
Zn Cl$_2$
D641
Zn SO$_4$
Zn Cl$_2$

TABLE 3.22 Influence of copper on yield measured as dry weight ($g\ l^{-1}$) in *E. mutabilis* in batch culture in 20 days at 25°C and photon flux density of 100 $\mu mol\ m^{-2}\ s^{-1}$; n = 16.

Cu ($mg\ l^{-1}$)		D464	D640	D641
Basal medium	$\bar{x}$	0.51	0.92	0.41
	sd	0.04	0.03	0.01
1.0	$\bar{x}$	0.34	0.47	0.31
	sd	0.03	0.01	0.01
2.5	$\bar{x}$	0.30	0.27	0.30
	sd	0.01	0.04	0.02
5.0	$\bar{x}$	0.24	0.22	0.27
	sd	0.02	0.04	0.01
10.0	$\bar{x}$	0.20	0.16	0.26
	sd	0.01	0.03	0.01
20.0	$\bar{x}$	0.16	0.12	0.20
	sd	0.03	0.01	0.01
40.0	$\bar{x}$	0.12	0.09	0.16
	sd	0.03	0.01	0.02

Fig. 3.19 Influence of Cu on yield in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 µmol $m^{-2}s^{-1}$


Cell density (cells ml^{-1} x 10^4)
30
10
0
0·001
0·01
0·1
1·0
10
Cu (mgl^{-1})
D464
D640
D641

3.74 Influence of manganese on yield

The effect of increased concentration of Mn on yield in *E. mutabilis* D464, D640 and D641 indicated no reduction in yield in all three strains in a concentration of 80 mg l^{-1} (Table 3.23).

3.75 Influence of iron on yield

Increasing the Fe concentration from 0.5 to 1.0 mg l^{-1} had no significant effect on yield. Further increase up to 7.5 reduced yield. Increased concentration from 7.5 to 10.0 brought about a total stoppage in growth in D464 and there was no growth in D640 and D641 above 5.0 mg l^{-1}. Cells were alive at 25 mg l^{-1} with no recovery at 50 mg l^{-1} after an exposure to this concentration in 20 days (Fig.3.20, Table 3.24).

3.76 Influence of aluminium on yield

An increase up to 1 mg l^{-1} Al brought about enhanced growth in all strains of *E. mutabilis*. Further increase up to 1000 mg l^{-1} had no significant increase in yield (Table 3.25).

3.77 Influence of lead on yield

With increased concentration of Pb in the medium (1.0 mg l^{-1}) Pb reduced yield in *E. mutabilis* D464, D640 and D641. Increasing the Pb concentration from 1.0 to 10.0 mg

TABLE 3.23 Influence of manganese on yield measured as dry weight ($g\ l^{-1}$) in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 $\mu mol\ m^{-2}\ s^{-1}$; n = 16.

Mn ($mg\ l^{-1}$)		D464	D640	D641
Basal medium (0.012)	$\bar{x}$	0.51	0.91	0.41
	sd	0.03	0.01	0.03
0.1	$\bar{x}$	0.52	0.92	0.42
	sd	0.03	0.03	0.03
1.0	$\bar{x}$	0.51	0.91	(.42
	sd	0.04	0.01	0.04
5.0	$\bar{x}$	0.50	0.92	0.41
	sd	0.04	0.03	0.01
10.0	$\bar{x}$	0.51	0.91	0.41
	sd	0.04	0.04	0.01
20.0	$\bar{x}$	0.50	0.92	0.40
	sd	0.02	0.04	0.03

TABLE 3.24 Influence of iron on yield measured as dry weight (g l^{-1}) in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}; n = 16.

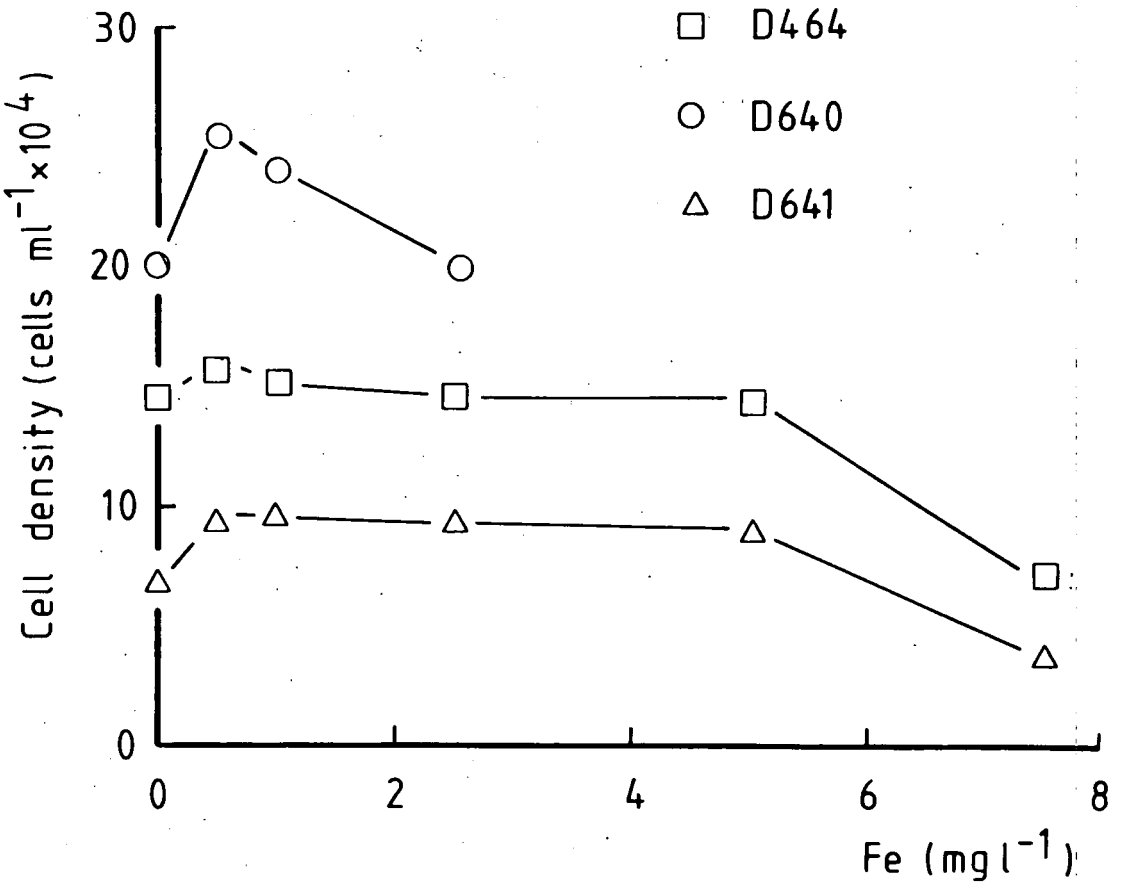
Fe (mg l^{-1})		D464	D640	D641
0	$\bar{x}$	0.41	0.87	0.39
	sd	0.02	0.04	0.01
0.5 (basal medium)	$\bar{x}$	0.52	0.91	0.41
	sd	0.03	0.01	0.04
1.0	$\bar{x}$	0.47	0.79	0.36
	sd	0.05	0.03	0.01
2.5	$\bar{x}$	0.31	0.48	0.23
	sd	0.04	0.02	0.01
5.0	$\bar{x}$	0.26		0.18
	sd	0.01		0.04
7.5	$\bar{x}$	0.17		0.13
	sd	0.03		0.02

TABLE 3.25 Influence of aluminium on yield measured as dry weight ($g\ l^{-1}$) in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 $\mu mol\ m^{-2}\ s^{-1}$; n = 16.

Al ($mg\ l^{-1}$)		D464	D640	D641
0	$\bar{x}$	0.51	0.92	0.41
	sd	0.04	0.02	0.01
1.0	$\bar{x}$	0.57	0.98	0.43
	sd	0.04	0.01	0.03
2.5	$\bar{x}$	0.53	0.95	0.43
	sd	0.02	0.04	0.01
5.0	$\bar{x}$	0.52	0.92	0.41
	sd	0.04	0.01	0.03
10.0	$\bar{x}$	0.52	0.92	0.41
	sd	0.03	0.04	0.01
100.0	$\bar{x}$	0.51	0.91	0.41
	sd	0.04	0.01	0.03
1000.0	$\bar{x}$	0.51	0.92	0.41
	sd	0.04	0.02	0.01

Fig. 3.20 Influence of Fe on yield in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 µmol $m^{-2}s^{-1}$

Fig. 3.21 Influence of Pb on yield in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 µmol $m^{-2}s^{-1}$


D464
D640
D641
Cell density (cells ml^{-1} x 10^4)
30
20
10
0
0
2
4
6
8
Fe (mg l^{-1})

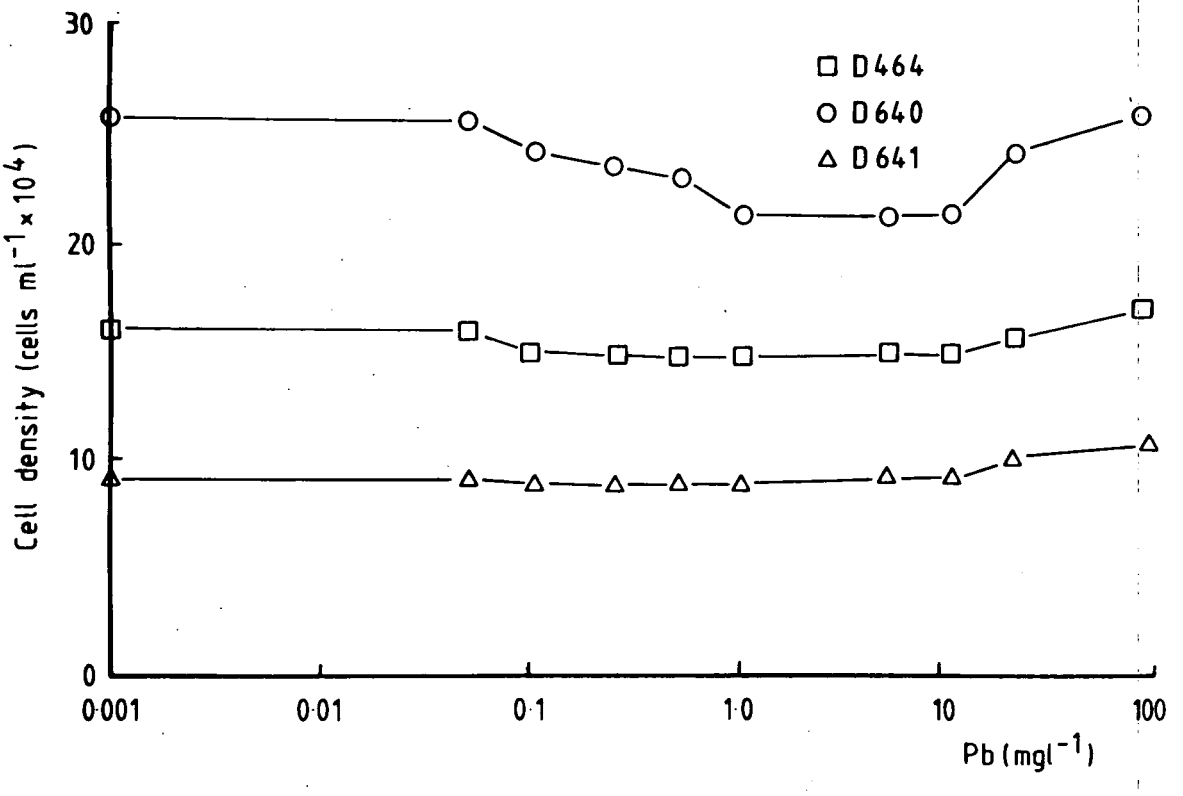

D464
D640
D641
Cell density (cells ml^{-1} x 10^4)
30
20
10
0
0·001
0·01
0·1
1·0
10
100
Pb (mgl^{-1})

TABLE 3.26 Influence of lead on yield measured as dry weight ($g\ l^{-1}$) in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 $\mu mol\ m^{-2}\ s^{-1}$; n=16.

Pb ($mg\ l^{-1}$)		D464	D640	D641
0.	$\bar{x}$	0.51	0.92	0.41
	sd	0.02	0.03	0.01
0.05	$\bar{x}$	0.50	0.90	0.39
	sd	0.04	0.01	0.03
0.1	$\bar{x}$	0.47	0.84	0.34
	sd	0.04	0.02	0.01
0.25	$\bar{x}$	0.44	0.72	0.30
	sd	0.04	0.01	0.03
0.5	$\bar{x}$	0.40	0.64	0.21
	sd	0.03	0.01	0.04
1.0	$\bar{x}$	0.40	0.64	0.23
	sd	0.04	0.01	0.03
5.0	$\bar{x}$	0.41	0.67	0.24
	sd	0.02	0.03	0.01
10.0	$\bar{x}$	0.42	0.67	0.25
	sd	0.02	0.04	0.01
20.0	$\bar{x}$	0.45	0.72	0.34
	sd	0.04	0.01	0.02
40.0	$\bar{x}$	0.53	0.86	0.38
	sd	0.03	0.05	0.01
80.0	$\bar{x}$	0.52	0.92	0.42
	sd	0.03	0.02	0.04
160.0	$\bar{x}$	0.53	0.93	0.43
	sd	0.01	0.04	0.02
200.0	$\bar{x}$	0.52	0.92	0.42
	sd	0.03	0.01	0.04

l^{-1} had no significant effect on yield (Fig. 3.22). Further increase from 10.0 to 100 brought about an increase in the yield (Table 3.26).

3.78 Influence of cadmium on yield

E. mutabilis D640 and D641 had no growth above 0.1 mg l^{-1} Cd, whereas D464 grew at 0.5 mg l^{-1}. Cells were alive at 25 mg l^{-1} and there was no recovery from exposure to 30 mg l^{-1} Cd (Fig. 3.22, Table 3.27).

3.79 Influence of nickel on yield

Increased Ni concentration from 0.0 to 0.25 mg l^{-1} brought about an increase in yield in *E. mutabilis* D464, D640 and D641, (Fig.3.23, Table 3.28). Further increase reduced yield. There was growth at and including 25 mg l^{-1} in strain D640 while D464 and D641 grew at 40 mg l^{-1}. All three strains recovered from exposure to 80 mg l^{-1} Ni.

3.710 Influence of cobalt on yield

E. mutabilis D464, D640 and D641 grew in the absence of added Co. Yield increased when subcultured in the basal medium (0.002 mg l^{-1} Co) compared with cobalt free medium (Table 3.29). There was growth at 40 mg l^{-1}Co and no recovery from exposure to 80 mg l^{-1} Co in all three strains.

3.8 Factors influencing zinc toxicity in *Euglena mutabilis* D464

3.81 Introduction

The influence of major cation and anions (Na, K, Mg, Ca, Cl, N), organic substrates (β-glycerophosphate,

TABLE 3.27 Influence of cadmium on yield measured as dry weight (g l^{-1}) in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}; n = 16.

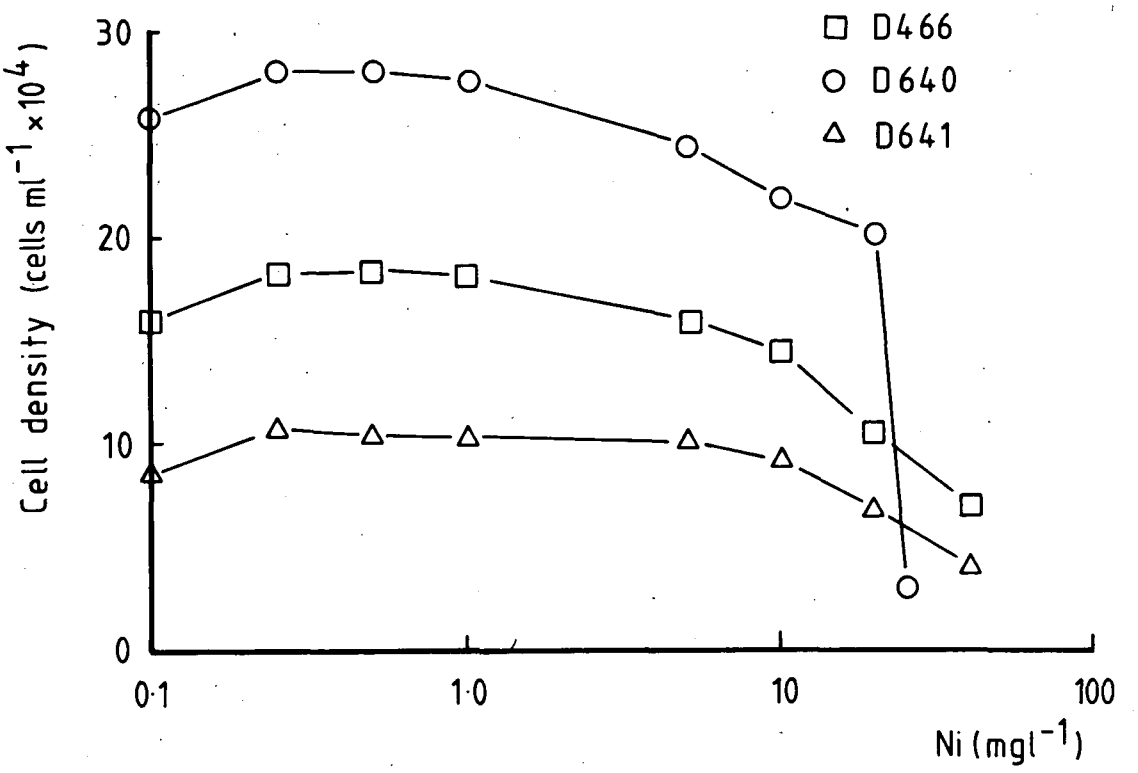
Cd (mg l^{-1})		D464	D640	D641
0	$\bar{x}$	0.51	0.92	0.41
	sd	0.03	0.01	0.04
0.005	$\bar{x}$	0.50	0.90	0.40
	sd	0.04	0.01	0.04
0.01	$\bar{x}$	0.50	0.87	0.36
	sd	0.03	0.01	0.04
0.1	$\bar{x}$	0.42	0.76	0.30
	sd	0.01	0.04	0.02
0.25	$\bar{x}$	0.37	0.62	0.23
	sd	0.04	0.02	0.01
0.5	$\bar{x}$	0.22	no growth	no growth
	sd	0.01		
1.0	no growth			

TABLE 3.28 Influence of nickel on yield measured as dry weight (g l^{-1}) in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}; n = 16.

Ni (mg l^{-1})		D464	D640	D641
0	$\bar{x}$	0.51	0.92	0.41
	sd	0.02	0.04	0.01
0.25	$\bar{x}$	0.54	0.95	0.44
	sd	0.03	0.01	0.03
0.5	$\bar{x}$	0.54	0.94	0.44
	sd	0.04	0.01	0.03
1.0	$\bar{x}$	0.53	0.93	0.43
	sd	0.03	0.01	0.04
5.0	$\bar{x}$	0.51	0.85	0.36
	sd	0.04	0.01	0.03
10.0	$\bar{x}$	0.46	0.81	0.32
	sd	0.03	0.01	0.03
20.0	$\bar{x}$	0.33	0.72	0.26
	sd	0.04	0.01	0.04
25.0	$\bar{x}$		0.34	
	sd		0.04	
40.0	$\bar{x}$	0.23		0.12
	sd	0.03		0.1

Fig. 3.22 Influence of Ni on yield in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 µmol $m^{-2}s^{-1}$

Fig. 3.23 Influence of Cd on yield in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 µmol $m^{-2}s^{-1}$


D466
D640
D641
Cell density (cells ml^{-1} x10^4)
30
20
10
0
0·1
1·0
10
100
Ni (mgl^{-1})

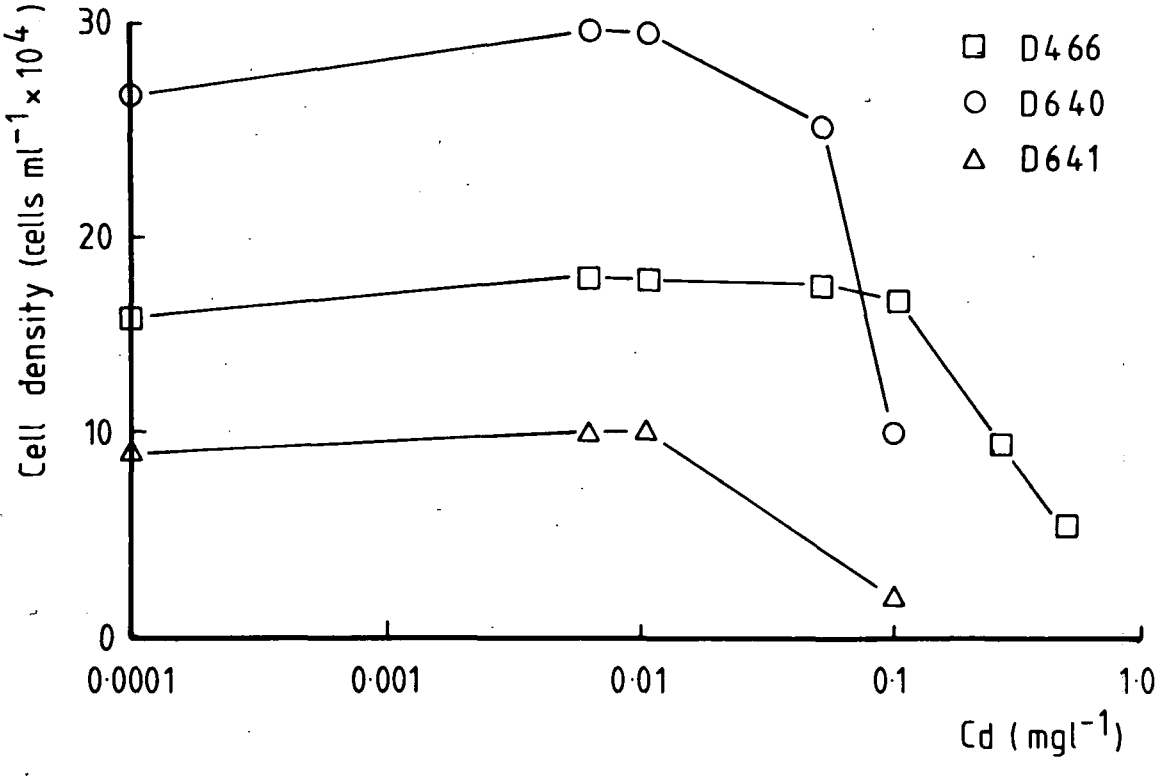

D466
D640
D641
Cell density (cells ml^{-1} x 10^4)
30
20
10
0
0·0001
0·001
0·01
0·1
1·0
Cd (mgl^{-1})

TABLE 3.29 Influence of cobalt on yield measured as dry weight (g l^{-1}) in *E. mutabilis* D464, D640 and D641 in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}; n = 16.

Co (mg l^{-1})		D464	D640	D641
0.002 (basal medium)	$\bar{x}$	0.51	0.92	0.41
	sd	0.02	0.02	0.01
0.25	$\bar{x}$	0.49	0.90	0.39
	sd	0.03	0.01	0.02
0.5	$\bar{x}$	0.47	0.90	0.37
	sd	0.04	0.01	0.03
1.0	$\bar{x}$	0.45	0.86	0.34
	sd	0.03	0.04	0.01
2.5	$\bar{x}$	0.43	0.86	0.30
	sd	0.04	0.01	0.01
5.0	$\bar{x}$	0.40	0.75	0.27
	sd	0.01	0.03	0.04
10.0	$\bar{x}$	0.36	0.71	0.25
	sd	0.04	0.01	0.01
20.0	$\bar{x}$	0.33	0.69	0.22
	sd	0.02	0.04	0.01
40.0	$\bar{x}$	0.16	0.24	0.11
	sd	0.04	0.1	0.03

glycyl-glycine) and other heavy metals (Cu, Mn, Fe, Al, Pb, Cd, Ni, Co) on zinc toxicity in *E. mutabilis* D464 was examined under standard growth conditions in sublethal (40 mg l^{-1}) and at inhibitory level (20 mg l^{-1}) Zn, using *E. mutabilis* D464. Inoculum for the experiments using organic substrates was from stock grown in the organic substrate under investigation. Each experiment was conducted four times with four replicates for each parameter under investigation.

3.82 Influence of inoculum size

With increased inoculum from 25 to 500 cells ml^{-1}, there was a reduction in zinc toxicity at sublethal Zn level (40 mg l^{-1}) in *E. mutabilis* D464 (Fig. 3.24, Table 3.30). A minimum of 10^{-2} cells ml^{-1} is necessary for a recognisable yield both as cell number and dry weight in 20 days of incubation (Section 2.4).

3.83 Influence of pH on zinc toxicity

With increased pH from 2.6 to 5.5 both at sublethal (40 mg l^{-1}) and inhibitory (20 mg l^{-1}) Zn levels, toxicity decreased up to and including pH 4.0. Further increase above pH 4.0, toxicity to Zn at both sublethal and inhibitory Zn levels increased. There was no yield above pH 5.5 (Fig. 3.25, Table 3.31).

3.84 Influence of major cations on zinc toxicity

The influence of major cations (Na, K, Mg, Ca) on zinc toxicity in *E. mutabilis* D464 was investigated under standard laboratory growth conditions (Section 2.4). Each

TABLE 3.30 Influence of inoculum size on sublethal (40 mg l^{-1}) Zn concentration in *E. mutabilis* D464, measured as dry weight (g l^{-1}) in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}; n = 16.

Inoculum size (cells ml^{-1})		dry weight (mg ml^{-1})
25	$\bar{x}$	0.21
	sd	0.01
50	$\bar{x}$	0.22
	sd	0.03
100	$\bar{x}$	0.51
	sd	0.03
200	$\bar{x}$	0.53
	sd	0.01
300	$\bar{x}$	0.50
	sd	0.04
400	$\bar{x}$	0.46
	sd	0.03
500	$\bar{x}$	0.44
	sd	0.03

Fig. 3.24 Influence of inoculum size on Zn toxicity in *E. mutabilis* in batch culture in 20 days at 25°C and photon flux density of 100 µmol $m^{-2}s^{-1}$

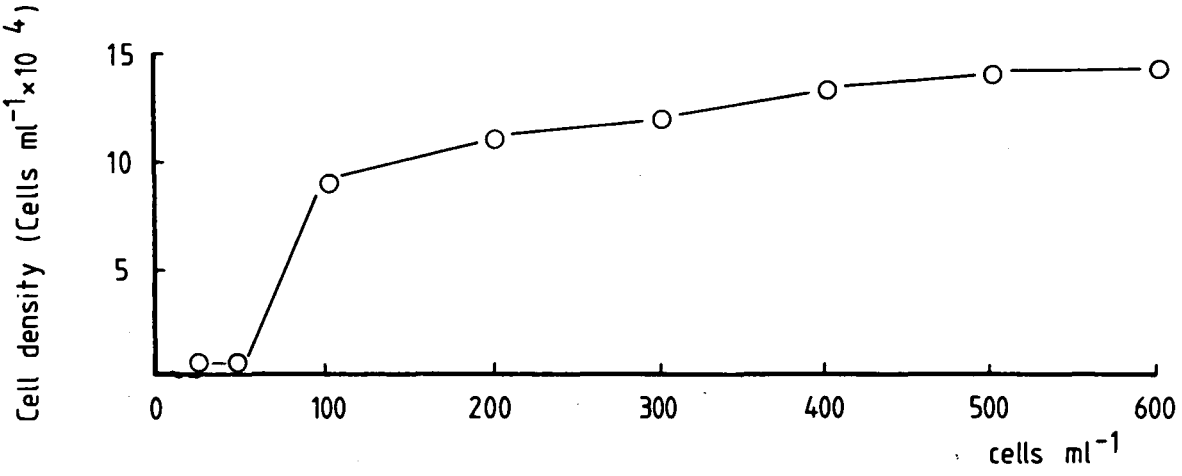
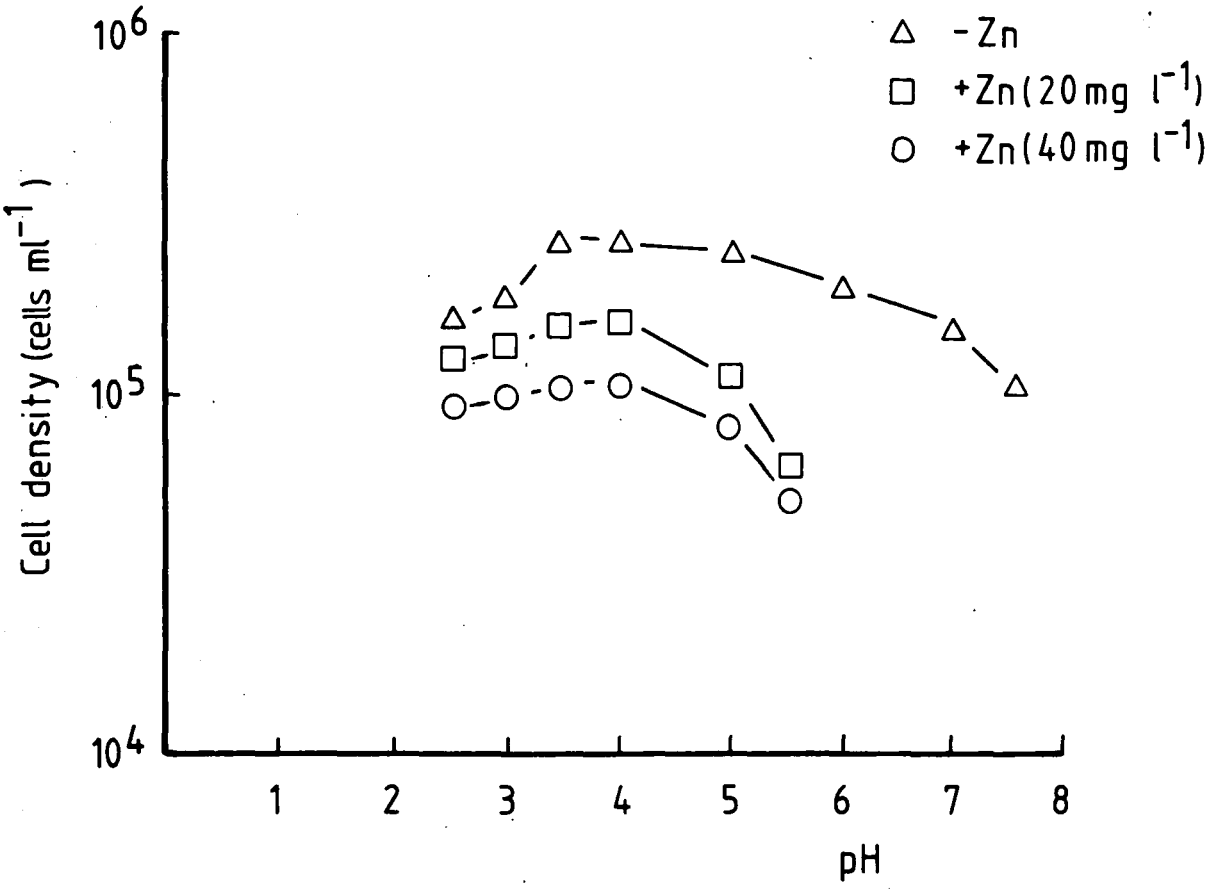

Cell density (Cells ml^{-1}x10^{4})
15
10
5
0
100
200
300
400
500
600
cells ml^{-1}

TABLE 3.3 1 Influence of pH on sublethal and inhibitory Zn levels in *E. mutabilis* D464 as dry weight ($g\ l^{-1}$) in 20 days in batch culture at 25°C and photon flux density of 100 $\mu mol\ m^{-2}\ s^{-1}$; n = 16.

pH		sublethal Zn level (40 $mg\ l^{-1}$)	inhibitory Zn level (20 $mg\ l^{-1}$)
2.6	$\bar{x}$	0.31	0.42
	sd	0.04	0.03
3.0	$\bar{x}$	0.33	0.44
	sd	0.03	0.01
3.4	$\bar{x}$	0.48	0.48
	sd	0.04	0.01
4.0	$\bar{x}$	0.48	0.48
	sd	0.04	0.03
5.0	$\bar{x}$	0.30	0.42
	sd	0.03	0.01
5.5	$\bar{x}$	0.22	0.31
	sd	0.01	0.04

Fig. 3.25 Influence of pH on Zn toxicity in *E. mutabilis* D464 in batch culture in 20 days at 25°C and photon flux density of 100 μmol m^{-2} s^{-1}


Cell density (cells ml^{-1})
10^6
10^5
10^4
1
2
3
4
5
6
7
8
pH
△ -Zn
□ +Zn(20 mg l^{-1})
○ +Zn(40 mg l^{-1})

experiment was carried out four times with four replicates for each concentration used.

3.841 Influence of sodium

Experiments conducted to check the influence of Na on zinc both at sublethal (40 mg l^{-1}) and inhibitory (20 mg l^{-1}) Zn levels indicated increased Zn toxicity with increased Na concentration (Fig.3.26, Table 3.32).

3.842 Influence of potassium

In the presence of sublethal Zn concentration, increasing the concentration of K from 2.5 to 5.0 mg l^{-1} brought about an increase in yield. Further increase from 5.0 to 160 mg l^{-1} had no significant effect on zinc toxicity at sublethal level (Table 3.33).

3.843 Influence of combined potassium and sodium on zinc toxicity

With 25 mg l^{-1} Na and 40 mg l^{-1} Zn, increased K concentration from 2.5 mg l^{-1} to 20 mg l^{-1}, reduced toxicity to Zn in *E. mutabilis*. Further increase from 20 mg l^{-1} to 100 mg l^{-1} reduced tolerance (Table 3.34).

3.844 Influence of magnesium

Increased Mg concentration to sublethal zinc level had no effect on either reducing or increasing Zn toxicity in *E. mutabilis* D464 over the range of concentrations of 10 to 160 mg l^{-1} Mg (Table 3.35).

Fig. 3.26 Influence of Na on Zn toxicity in *E. mutabilis* D464 in batch culture in 20 days at 25°C and photon flux density of 100 μmol m^{-2} s^{-1}

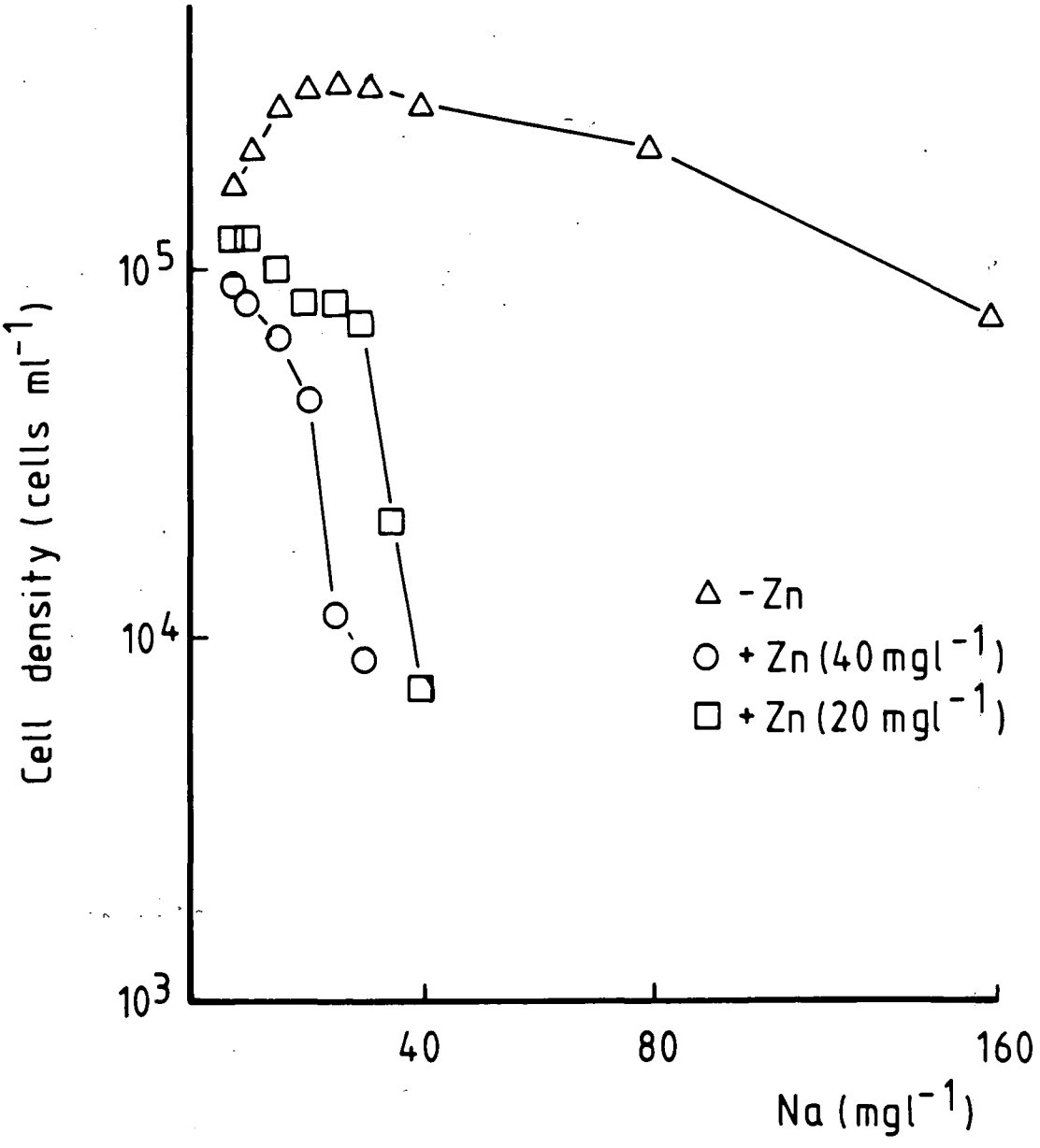

Cell density (cells ml^{-1})
10^5
10^4
10^3
40
80
160
Na (mgl^{-1})
△ - Zn
○ + Zn (40 mgl^{-1})
□ + Zn (20 mgl^{-1})

TABLE 3.32 Influence of sodium on sublethal and inhibitory Zn levels in *E. mutabilis* D464 measured as dry weight (g l^{-1}) in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}; n = 16.

Na (mg l^{-1})		sublethal Zn level	inhibitory Zn level
7 (basal medium)	$\bar{x}$	0.31	0.42
	sd	0.04	0.01
10	$\bar{x}$	0.27	0.40
	sd	0.01	0.04
15	$\bar{x}$	0.27	0.38
	sd	0.04	0.03
20	$\bar{x}$	0.24	0.32
	sd	0.03	0.01
25	$\bar{x}$	0.20	0.31
	sd	0.04	0.03
30	$\bar{x}$	0.13	0.31
	sd	0.04	0.02
35	$\bar{x}$	no growth	0.25
	sd		0.03
40	$\bar{x}$		0.12
	sd		0.04

TABLE 3.33 Influence of potassium on Zn toxicity at sublethal Zn level in *E. mutabilis* D464 measured as dry weight ($g\ l^{-1}$) in batch culture in 20 days at 25°C and photon flux density of 100 $\mu mol\ m^{-2}\ s^{-1}$; n = 16.

K ($mg\ l^{-1}$)	dry weight ($g\ l^{-1}$)	
2.5 (basal medium)	$\bar{x}$	0.31
	sd	0.04
5.0	$\bar{x}$	0.33
	sd	0.03
10.0	$\bar{x}$	0.33
	sd	0.04
20.0	$\bar{x}$	0.33
	sd	0.03
40.0	$\bar{x}$	0.34
	sd	0.04
80.0	$\bar{x}$	0.33
	sd	0.02
160.0	$\bar{x}$	0.33
	sd	0.02

TABLE 3.34 Influence of combined sodium and potassium on zinc toxicity in *E. mutabilis* D464 measured as dry weight ($g\ l^{-1}$) in batch culture in 20 days at 25°C and photon flux density 100 µmol $m^{-2}\ s^{-1}$; n = 16.

K ($mg\ l^{-1}$)		sublethal Zn level plus 25 $mg\ l^{-1}$ Na
2.5	$\bar{x}$	0.21
	sd	0.01
5.0	$\bar{x}$	0.22
	sd	0.04
10.0	$\bar{x}$	0.25
	sd	0.02
20.0	$\bar{x}$	0.26
	sd	0.04
30.0	$\bar{x}$	0.24
	sd	0.03
40.0	$\bar{x}$	0.24
	sd	0.03
50.0	$\bar{x}$	0.22
	sd	0.03
60.0	$\bar{x}$	0.20
	sd	0.02
80.0	$\bar{x}$	0.18
	sd	0.02
100.0	$\bar{x}$	0.15
	sd	0.02

TABLE 3.35 Influence of magnesium on zinc toxicity in *E. mutabilis* D464 measured as dry weight ($g\ l^{-1}$) in batch culture in 20 days at 25°c and photon flux density of 100 $\mu mol\ m^{-2}\ s^{-1}$; n = 16.

Mg ($mg\ l^{-1}$)		dry weight ($g\ l^{-1}$)
2.5	$\bar{x}$	0.31
	sd	0.02
5.0	$\bar{x}$	0.33
	sd	0.02
10.0	$\bar{x}$	0.35
	sd	0.02
20.0	$\bar{x}$	0.35
	sd	0.02
40.0	$\bar{x}$	0.34
	sd	0.04
80.0	$\bar{x}$	0.34
	sd	0.03
160.0	$\bar{x}$	0.34
	sd	0.02
200.0	$\bar{x}$	0.35
	sd	0.02

3.845 Influence of calcium

Experiments conducted to see the effect of increased Ca concentration had on zinc toxicity at sublethal level (40 mg l^{-1}) and at inhibitory level (20 mg l^{-1}) Zn, indicated increased toxicity with increased Ca concentration above 10 mg l^{-1} with no yield above 30 mg l^{-1} with sublethal and 60 mg l^{-1} inhibitory level of zinc (Fig. 3.27, Table 3.36).

3.85 Influence of anions on zinc toxicity

3.851 Influence of chloride

Increasing the Cl concentration from 35 to 70 mg l^{-1} reduced toxicity to sublethal level of Zn in *E. mutabilis* D464. Further increase from 70 to 90 mg l^{-1} Cl reduced tolerance (Table 3.37).

3.852 Influence of ammonium-nitrogen

Increasing the concentration of ammonium-nitrogen in the basal medium in the presence of sublethal zinc levels, had no significant effect on Zn toxicity (Table 3.38).

Fig. 3.27 Influence of Ca on Zn toxicity in *E. mutabilis* D464 in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}

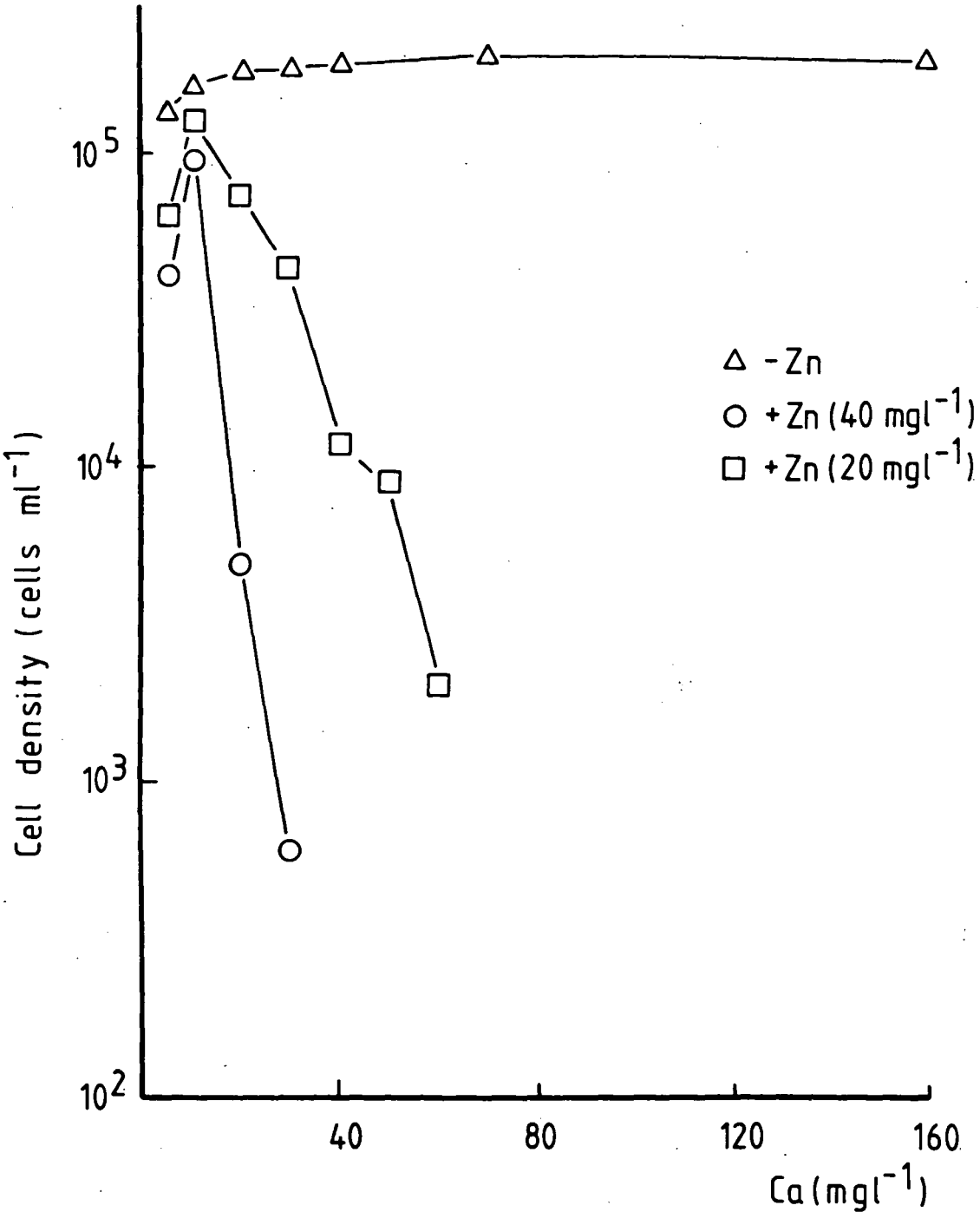

Cell density (cells ml^{-1})
10^5
10^4
10^3
10^2
40
80
120
160
Ca (mgl^{-1})
△ - Zn
○ + Zn (40 mgl^{-1})
□ + Zn (20 mgl^{-1})

TABLE 3.3 6 Influence of calcium on zinc toxicity in *E. mutabilis* D464 measured as dry weight ($g\ l^{-1}$) in batch culture in 20 days at 25°C and photon flux density 100 µmol m^{-2} s^{-1}; n = 16.

Ca ($mg\ l^{-1}$)		dry weight ($g\ l^{-1}$)	
		sublethal Zn level	inhibitory Zn level
5.0	$\bar{x}$	0.24	0.30
	sd	0.02	0.01
10.0	$\bar{x}$	0.31	0.41
	sd	0.04	0.03
20.0	$\bar{x}$	0.21	0.33
	sd	0.01	0.04
30.0	$\bar{x}$	0.11	0.22
	sd	0.03	0.01
40.0	$\bar{x}$	no growth	0.16
	sd		0.03
50.0	$\bar{x}$		0.12
	sd		0.03
60.0	$\bar{x}$		0.11
	sd		0.04

TABLE 3.37 Influence of chloride on Zn toxicity in *E. mutabilis* D464 measured as dry weight ($g\ l^{-1}$) in batch culture in 20 days at 25°C and photon flux density of 100 µmol $m^{-2}\ s^{-1}$; n = 16.

Cl ($mg\ l^{-1}$)		dry weight ($g\ l^{-1}$)
35.0	$\bar{x}$	0.31
	sd	0.03
40.0	$\bar{x}$	0.31
	sd	0.04
50.0	$\bar{x}$	0.32
	sd	0.03
60.0	$\bar{x}$	0.32
	sd	0.01
70.0	$\bar{x}$	0.31
	sd	0.04
80.0	$\bar{x}$	0.32
	sd	0.03
90.0	$\bar{x}$	0.31
	sd	0.01

TABLE 3.38 Influence of ammonium-nitrogen on Zn toxicity in *E. mutabilis* D464 measured as dry weight in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}; n = 16.

N (mg l^{-1})		dry weight (g l^{-1})
10.0	$\bar{x}$	0.31
	sd	0.03
20.0	$\bar{x}$	0.31
	sd	0.04
25.0	$\bar{x}$	0.30
	sd	0.04
30.0	$\bar{x}$	0.31
	sd	0.03
35.0	$\bar{x}$	0.32
	sd	0.01
40.0	$\bar{x}$	0.31
	sd	0.03
50.0	$\bar{x}$	0.31
	sd	0.02
60.0	$\bar{x}$	0.32
	sd	0.03
70.0	$\bar{x}$	0.32
	sd	0.04
80.0	$\bar{x}$	0.32
	sd	0.02
160.0	$\bar{x}$	0.31
	sd	0.03

3.86 Influence of organic substrates on zinc toxicity

3.861 Influence of glycyl-glycine

In the presence of sublethal zinc level (40 mg l^{-1}) increased concentration of glycyl-glycine nitrogen had no effect on Zn toxicity (Table 3.39).

3.862 Influence of β-glycerophosphate

With increased β-glycerophosphate-P, there was increased toxicity with increased P (Table 3.40).

3.87 Influence of other heavy metals on zinc toxicity

3.871 Influence of copper

With increased Cu in the growth medium, both at sublethal (40 mg l^{-1}) and inhibitory level (20 mg l^{-1}) of Zn, yield decreased with increased Cu levels (Table 3.41).

3.872 Influence of manganese

The results (Table 3.42) of the experiments conducted to check the influence of Mn on sublethal Zn level, indicate no effect on zinc toxicity.

3.873 Influence of iron

Increasing the Fe concentration to both sublethal and inhibitory level of Zn, increased Zn toxicity with no yield in the sublethal level at 1 mg l^{-1} Fe (Fig. 3.28, Table 3.43).

TABLE 3.39 Influence of glycyl-glycine-nitrogen on Zn toxicity in *E. mutabilis* D464 measured as dry weight in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}; n = 16.

glycyl-glycine-nitrogen (mg l^{-1})		dry weight (g l^{-1})
5.0	$\bar{x}$	0.30
	sd	0.01
10.0	$\bar{x}$	0.31
	sd	0.04
20.0	$\bar{x}$	0.34
	sd	0.04
30.0	$\bar{x}$	0.34
	sd	0.03
35.0	$\bar{x}$	0.35
	sd	0.03
40.0	$\bar{x}$	0.36

TABLE 3.40 Influence of β-glycerophosphate on Zn toxicity in *E. mutabilis* D464 in batch culture measured as dry weight in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}; n = 16.

β-glycerophosphate (mg l^{-1})		dry weight (g l^{-1})
1.8	$\bar{x}$	0.24
	sd	0.01
2.5	$\bar{x}$	0.23
	sd	0.04
5.0	$\bar{x}$	0.21
	sd	0.03
10.0	$\bar{x}$	0.20
	sd	0.03
20.0	$\bar{x}$	0.17
	sd	0.03
30.0	$\bar{x}$	0.13
	sd	0.01

TABLE 3.41 Influence of Cu on sublethal and inhibitory Zn levels in *E. mutabilis* D464 measured as dry weight in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}; n = 16.

Cu (mg l^{-1})		sublethal Zn level	inhibitory Zn level
0.004	$\bar{x}$	0.31 mg ml^{-1}	0.41 g l^{-1}
	sd	0.01	0.04
0.01	$\bar{x}$	0.22	0.34
	sd	0.01	0.04
0.05	$\bar{x}$	0.17	0.22
	sd	0.03	0.01
0.1	$\bar{x}$	0.12	0.16
	sd	0.02	0.04
0.25	$\bar{x}$	0.01	0.12
	sd	0.04	0.02
0.5	$\bar{x}$	no growth	0.1
	sd		0.04

TABLE 3.42 Influence of Mn on sublethal Zn levels in *E. mutabilis* D464 measured as dry weight (g l^{-1}) in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}; n = 16.

Mn (mg l^{-1})		dry weight (g l^{-1})
0.012	$\bar{x}$	0.31
	sd	0.03
1.0	$\bar{x}$	0.31
	sd	0.04
5.0	$\bar{x}$	0.31
	sd	0.01
10.0	$\bar{x}$	0.31
	sd	0.04
20.0	$\bar{x}$	0.31
	sd	0.01
40.0	$\bar{x}$	0.31
	sd	0.04
80.0	$\bar{x}$	0.32
	sd	0.01

Fig. 3.28 Influence of Fe on Zn toxicity in *E. mutabilis* D464 in batch culture in 20 days at 25°C and photon flux density of 100 μmol m^{-2} s^{-1}

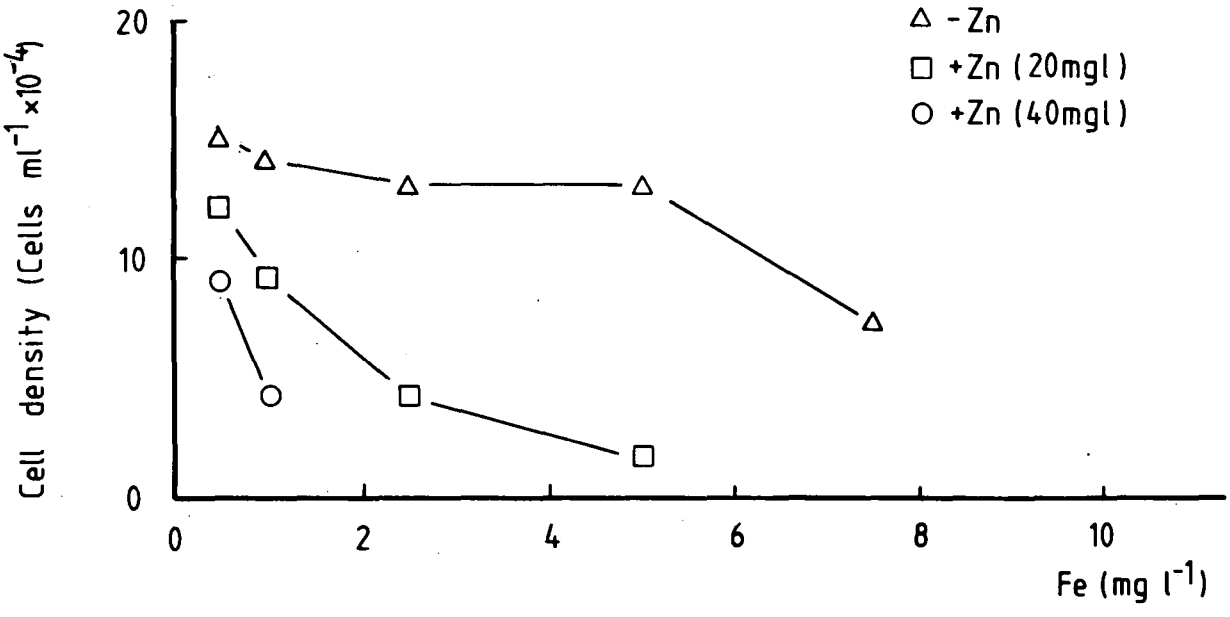

Cell density (Cells ml^{-1} x10^{-4})
20
10
0
0
2
4
6
8
10
Fe (mg l^{-1})
△ - Zn
□ +Zn (20mgl)
○ +Zn (40mgl)

TABLE 3.43 Influence of Fe on sublethal and inhibitory Zn levels in *E. mutabilis* D464 measured as dry weight in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}; n=16.

Fe (mg l^{-1})		sublethal Zn level	inhibibitory Zn level
0.5 (basal medium)	$\bar{x}$	0.32 g l^{-1}	0.41 g l^{-1}
	sd	0.01	0.04
1.0	$\bar{x}$	0.20	0.31
	sd	0.02	0.01
2.5	$\bar{x}$	no growth	0.22
	sd		0.04
5.0	$\bar{x}$		0.17
	sd		0.04

3.874 Influence of aluminium

Increasing the Al concentration from 1 to 100 mg l^{-1}, in the presence of sublethal Zn levels, there was no significant change in the tolerance to Zn. Further increase from 100 to 1000 mg l^{-1} Al increased toxicity to sublethal Zn concentration (Table 3.44).

3.875 Influence of lead

Results of the experiments conducted to see the effect of increasing the concentration of Pb to sublethal and inhibitory level of Zn indicated that with increased Pb toxicity to zinc increased (Fig. 3.29, Table 3.45).

3.876 Influence of cadmium

With increased Cd concentration from 0 to 0.1 mg l^{-1} increased Zn toxicity, with no yield above 0.1 mg l^{-1} Cd at sublethal Zn level (Fig. 3.30, Table 3.46).

TABLE 3.44 Influence of Al on sublethal levels in *E. mutabilis* D464 measured as dry weight ($g\ l^{-1}$) in batch culture in 20 days at 25°C and photon flux density of 100 $\mu mol\ m^{-2}\ s^{-1}$; n = 16.

Al ($mg\ l^{-1}$)		dry weight ($g\ l^{-1}$)
0.	$\bar{x}$	0.31
	sd	0.01
1.0	$\bar{x}$	0.32
	sd	0.03
2.5	$\bar{x}$	0.32
	sd	0.01
5.0	$\bar{x}$	0.33
	sd	0.04
10.0	$\bar{x}$	0.32
	sd	0.04
50.0	$\bar{x}$	0.33
	sd	0.03
100.0	$\bar{x}$	0.32
	sd	0.03
200.0	$\bar{x}$	0.30
	sd	0.02
250.0	$\bar{x}$	0.26
	sd	0.04
500.0	$\bar{x}$	0.22
	sd	0.03
1000.0	$\bar{x}$	0.20
	sd	0.01

Fig. 3.29 Influence of Pb on Zn toxicity in *E. mutabilis* D464 in batch culture in 20 days at 25°C and photon flux density of 100 μmol m^{-2} s^{-1}

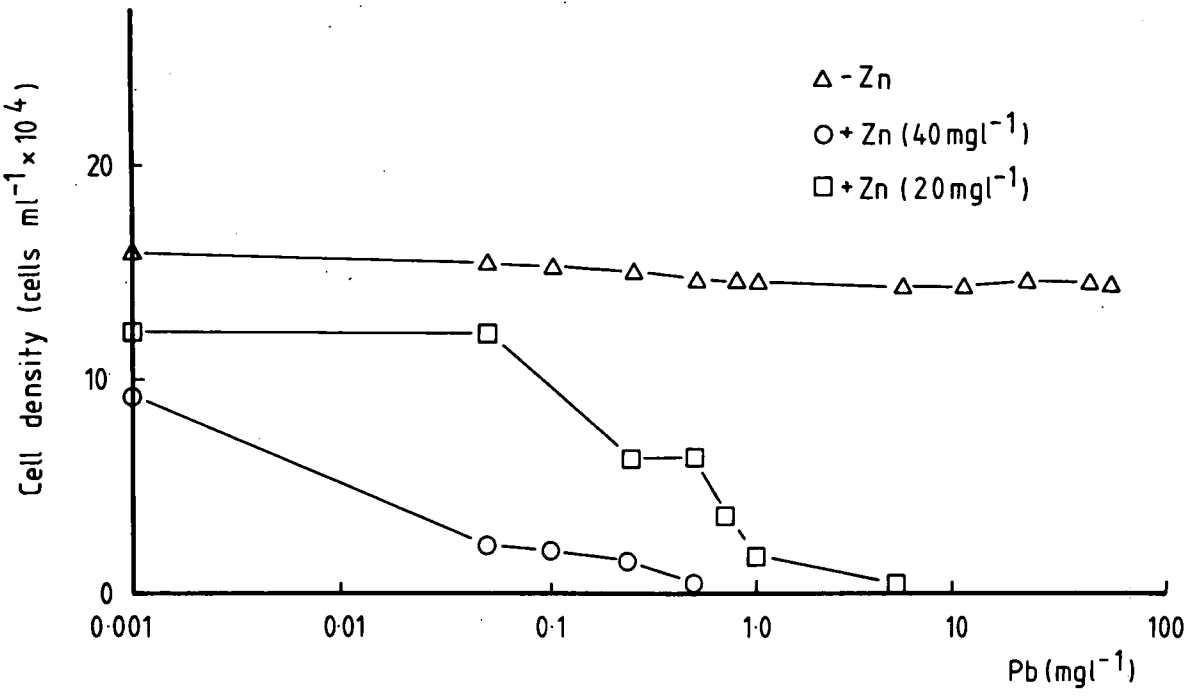

Cell density (cells $ml^{-1} \times 10^4$)
20
10
0
0·001
0·01
0·1
1·0
10
100
Pb (mgl^{-1})
△ - Zn
○ + Zn (40 mgl^{-1})
□ + Zn (20 mgl^{-1})

TABLE 3.45 Influence of Pb on sublethal and inhibitory Zn levels in *E. mutabilis* D464 measured as dry weight in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}; n=16.

Pb (mg l^{-1})		sublethal Zn level	inhibitiory Zn level
0.0	$\bar{x}$	0.31 g l^{-1}	0.41 g l^{-1}
	sd	0.03	0.01
0.05	$\bar{x}$	0.22	0.41
	sd	0.01	0.04
0.1	$\bar{x}$	0.20	0.40
	sd	0.04	0.01
0.25	$\bar{x}$	0.17	0.40
	sd	0.04	0.01
0.5	$\bar{x}$	0.13	0.38
	sd	0.02	0.04
0.75	$\bar{x}$	no growth	0.34
	sd		0.02
1.0	$\bar{x}$		0.31
	sd		0.03
1.25	$\bar{x}$		0.27
	sd		0.03
1.5	$\bar{x}$		0.12
	sd		0.02

Fig. 3.30 Influence of Cd on Zn toxicity in *E. mutabilis* D464 in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}

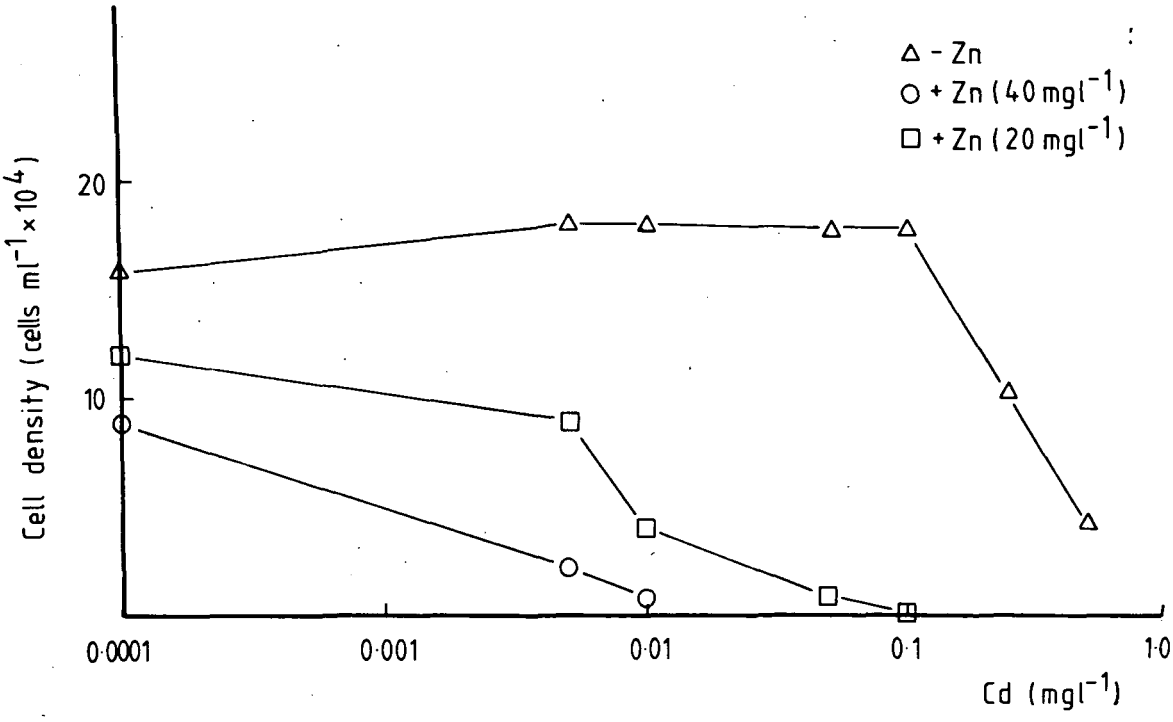

△ - Zn
○ + Zn (40 mgl^{-1})
□ + Zn (20 mgl^{-1})
Cell density (cells ml^{-1} x 10^4)
20
10
0·0001
0·001
0·01
0·1
1·0
Cd (mgl^{-1})

TABLE 3.46 Influence of Cd on sublethal and inhibitory Zn levels in *E. mutabilis* D464 measured as dry weight ($g\ l^{-1}$) in batch culture in 20 days at 25°C and photon flux density of 100 $\mu mol\ m^{-2}\ s^{-1}$; n = 16

Cd ($mg\ l^{-1}$)		sublethal Zn level	inhibitory Zn level
0.0	$\bar{x}$	0.31 $g\ l^{-1}$	0.41 $g\ l^{-1}$
	sd	0.02	0.01
0.005	$\bar{x}$	0.17	0.31
	sd	0.04	0.03
0.01	$\bar{x}$	0.12	0.22
	sd	0.01	0.04
0.05	$\bar{x}$	no growth	0.17
	sd		0.03
0.1	$\bar{x}$		0.13
	sd		0.03

3.877 Influence of nickel

Increasing the nickel concentration above 0.25 to 5.0 mg l^{-1} in the presence of both sublethal and inhibitory Zn levels, increased Zn toxicity (Table 3.47).

3.878 Influence of cobalt

Increased Co concentration from 0.002 mg l^{-1} to 5.0 mg l^{-1} there was no change in Zn toxicity whereas above 5 mg l^{-1} Cd, reduced toxicity to Zn (Fig.3.31; Table 3.48).

TABLE 3.47 Influence of Ni on sublethal and inhibitory Zn levels in *E. mutabilis* D464 measured as dry weight ($g\ l^{-1}$) in batch culture in 20 days at 25°C and photon flux density of 100 $\mu mol\ m^{-2}\ s^{-1}$; n = 16.

Ni ($mg\ l^{-1}$)		sublethal Zn level	inhibitory Zn level
0.0	$\bar{x}$	0.31 $g\ l^{-1}$	0.41 $g\ l^{-1}$
	sd	0.04	0.01
0.25	$\bar{x}$	0.27	0.41
	sd	0.04	0.03
0.5	$\bar{x}$	0.25	0.41
	sd	0.04	0.03
1.0	$\bar{x}$	0.23	0.38
	sd	0.03	0.01
5.0	$\bar{x}$	0.22	0.44
	sd	0.01	0.04
10.0	$\bar{x}$	0.17	0.27
	sd	0.02	0.04
20.0	$\bar{x}$	0.17	0.23
	sd	0.03	0.01
40.0	$\bar{x}$	0.12	0.17
	sd	0.03	0.04

Fig. 3.31 Influence of Co on Zn toxicity in *E. mutabilis* D464 in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}

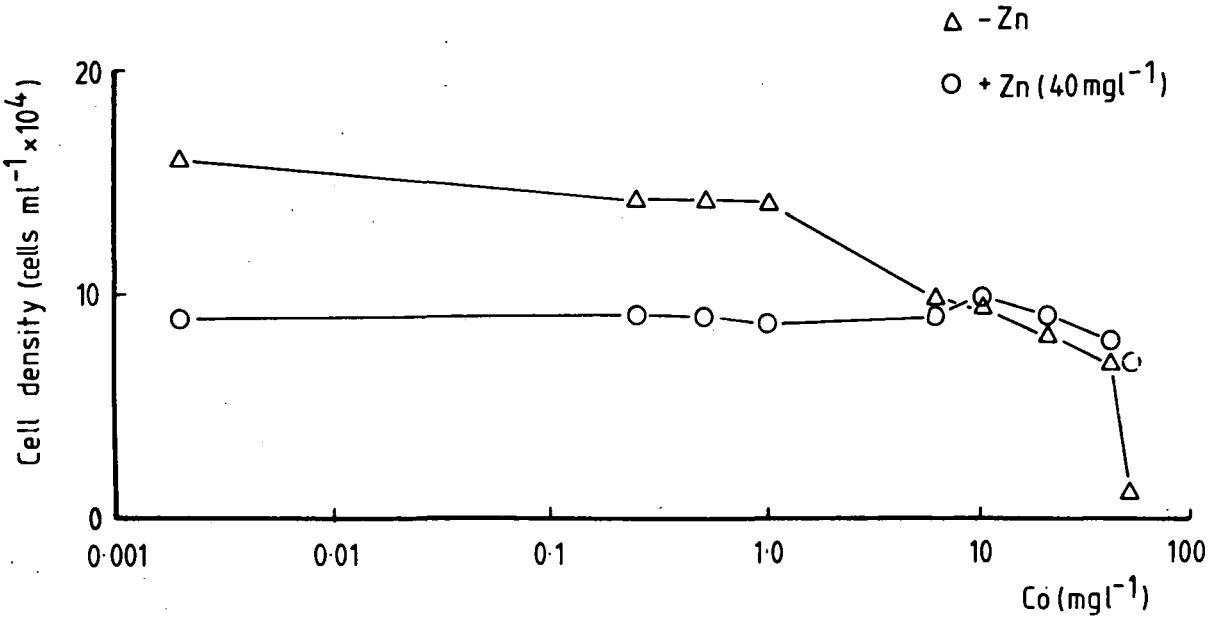

Δ - Zn
O + Zn (40 mgl^{-1})
Cell density (cells ml^{-1} x 10^4)
20
10
0
0·001
0·01
0·1
1·0
10
100
Co (mgl^{-1})

TABLE 3.48 Influence of Co on sublethal Zn levels in *E. mutabilis* D464 measured as dry weight (g l^{-1}) in batch culture in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}; n = 16.

Co (mg l^{-1})		dry weight (g l^{-1})
0.002	$\bar{x}$	0.31
	sd	0.03
0.25	$\bar{x}$	0.32
	sd	0.02
0.5	$\bar{x}$	0.32
	sd	0.03
1.0	$\bar{x}$	0.31
	sd	0.04
5.0	$\bar{x}$	0.32
	sd	0.04
10.0	$\bar{x}$	0.31
	sd	0.01
20.0	$\bar{x}$	0.32
	sd	0.02
40.0	$\bar{x}$	0.26
	sd	0.03
50.0	$\bar{x}$	0.22
	sd	0.02

CHAPTER FOUR

DISCUSSION

4.1 Influence of pH

Most algae in aquatic habitats are found in near neutral pH with a few at extreme pH limits. In acid environments, one of the problems that acid algae have to surmount is associated with hydrogen ions causing depletion of essential nutrients from the microenvironment (Section 1.2). The pH range at which algae occur may vary, with some low pH thermophilic species growing at extreme low pH (Brock, 1972). Fott and McCarthy (1964) isolated *Chlamydomonas acidophila* from a peat bog at pH 1.0 and Hargreaves *et al.* (1975) *Euglena mutabilis* from pH 1.5. In the current research four strains of *E. mutabilis*, D464, D640, D641 and D642 were isolated from acid environments with pH 2.6, 1.9, 3.5 and 3.7, respectively. Laboratory investigations indicate that all four strains have a wide range of pH tolerances, with an optimum yield between pH 3.4 and 4.0. This is close to the findings of Dach (1943) where he reported that *E. mutabilis* had a total range of growth from pH 2.1 to 7.9 with a maximum growth between pH 3.4 and 5.4.

It was possible to adapt *E. mutabilis* D464, isolated from and maintained at pH 2.6, to grow to pH 1.5 and 8.5 by continuous subculture in very low (pH 1.8) and high (pH 7.6) pH for six months.

Dach (1943) reported that *E. mutabilis* survived for at least 12 days when grown in organic medium over a range of pH values of pH 1.4 to 7.9. The author found that at pH 1.0 it survived for 6 hours and at pH 1.4 for 4 days. In the current investigation it was found that *E. mutabilis* D464, in

inorganic basal medium could survive for 6 hours in pH 1.0 and for four days in pH 1.2. There was recovery from exposure to pH 1.4 even after 20 days.

The presence of *E. mutabilis* in acid environments (exception being its absence from Kootenay Paint Pots, British Columbia, Wehr and Whitton, 1983), may have some relevance to an internal pH mechanism that may be in operation. Lane and Burris (1981) found that *E. mutabilis* has a different internal pH to that of the external environment. The authors found that its internal pH is below pH 6.0 at low external pH and that with external pH rising above 6.0, *E. mutabilis* was not able to maintain its internal pH. If maintaining a steady internal pH is an energy requiring process, then the cost of maintaining the internal pH may drain valuable energy which is otherwise used for growth and reproduction.

4.2 Influence of Nutrients

Euglena mutabilis, D464, D640 and D641 were subjected to different concentrations of major cations and anions in the growth media, under standard laboratory conditions. It was found that there was improved yield with increased Na concentration. Allen (1952) reported that *Anabaena cylindrica* required 'appreciable' concentrations of Na for better growth. Increased K concentration from 2.5 mg l^{-1} to 5.0 mg l^{-1} improved yield in *E. mutabilis* with no effect above 5.0 mg l^{-1}. There was no growth in the absence of Mg and Ca. Retovsky and Klasterska (1961) observed that *Chlorella* cells became 'chlorotic, enlarged and extensively vaculated' in the absence to Mg. In the current investigation, in the absence of Mg, *E. mutabilis*

became enlarged and dark brown in colour. Chang and Kahn (1966) reported that in *E. gracilis* there is a Mg dependent ATPase and a Ca dependent ATPase. It is probable that a similar system may be in operation in *E. mutabilis*.

Of the different inorganic nitrogen sources for algal nutrition, nitrates and ammonium ions are the most common. Though some use both nitrates and ammonium-nitrogen, some use ammonium-N preferentially when supplied with both nitrates and ammonium ions as nitrogen source (Syrett, 1962). Sakari (1976) noted that in a pH range of 4.6 to 5.1, the greater part of the total nitrogen consists of NH_4 and nitrogen in the form of nitrates become unavailable at low pH. With all three strains of *E. mutabilis* tested, there was growth only in ammonium-N and not in nitrates. Continous subculture in the basal medium with nitrate-N did not induce growth in all three strains. Similar findings with *E. gracilis* (Neilson and Larsson, 1980) and in 12 strains of *Chlorella protothecoides* (Albertano and Taddei, 1978), where the algae were unable to utilize nitrates have been reported. Moss (1973) found that most Euglenophyta tested in cultures seem unable to utilize nitrates.

With the micronutrients (Zn, Cu, Mn, Co) tested, Zn brought about an increase in yield even when supplied in macro level (10 mg l^{-1}). This confirms Hargreaves and Whitton (1976) who found similar increase in growth with increased Zn concentration. This increase in growth with added Zn may be due to Zn being used in the protein synthesis as hypothesised by Prask and Plocke (1971) or as Keilin and Mann (1940) to be involved with CO_2 fixation. The latter seems to be a more probable explanation as CO_2 levels (Section 1.3) are low at low pH.

Green and co-workers (1939) demonstrated that there is a direct participation of Cu in photosynthesis in *Chlorella pyrenoidosa* where the addition of organic compounds which form complexes with Cu, resulted in a reversible decrease in photosynthetic activity. With concentration of Cu falling below 10^{-7}M Walker (1953) found deficiency symptoms in *Chlorella* sp. while Greenfield (1942) found toxic effects of Cu at this concentration in *C. vulgaris*. In the current investigation Cu as 'impurities' (>0.002 mg l^{-1}) brought about measurable yield which increased with Cu concentration up to and including 0.005 mg l^{-1}. Further increase up to 1.0 mg l^{-1} reduced yield.

Manganese has been shown to be a requirement for algal growth (Harvey, 1947) in photosynthesis, in respiration, in nitrogen metabolism and in relation to other elements in the growth medium (Wiessner, 1962). Harvey found that Mn was necessary for 'vigorous' growth in "*Chlamydomonas*" and a marine *Cryptomonas*. Walker (1954) found similar results with *Chlorella pyrenoidosa*. Brown (1954) demonstrated that Hill Reaction is completely suppressed in the absence of Mn. Hewitt (1958) found respiration was reduced in *C. pyrenoidosa* and *Ankistrodesmus braunii* in the absence of Mn. In nitrogen metabolism, Noack and Pirson (1939) reported that the rate of growth in *C. pyrenoidosa* is reduced in the absence of Mn whether nitrate or an ammonium salt is used as nitrogen source. Waren (1933) found an antagonism between Mn and Ca in *Micrasterias rotata*, where Mn increased cell division in high concentration of Ca and inhibited cell division in low concentration of Ca. Pirson (1939) reported an antagonism between Mn and K, where at low concentration of K, *C. pyrenoidosa*

required low concentration of Mn whereas with high concentration of K it required higher concentration of Mn for healthy growth. Although *E. mutabilis* was able to grow in Mn free basal medium (except for Mn as 'impurities') its addition (0.012 mg l^{-1}) improved yield. Any further addition from 0.012 to 1.0 mg l^{-1} Mn to the basal medium had no effect on yield.

Krauss (1955) reported that cobalt stimulated growth in *Scenedesmus obliquus* and Holm-Hansen *et al.*,(1954) showed the importance of Co to a number of blue-green algae and its replaceability by vitamin B_{12}. When *E. mutabilis* grown in cobalt free medium was subcultured in the basal medium with 0.002 mg l^{-1}, Co yield was improved. A similar result was seen when cells were subcultured in 1 mg l^{-1} vitamin B_{12}. As Co is a known constituent of vitamin B_{12}, it was hypothesised that the improved yield seen in the presence of cobalt was due to its incorporation into vitamin B_{12}. But experiments carried out with Co and vitamin B_{12} showed improved yield in the presence of vitamin B_{12} without Co than with Co without vitamin B_{12}. As no check was made to see if cultures grown in the presence of Co had any vitamin B_{12} at the end of the incubation period, it is not possible to state whether *E. mutabilis* is a vitamin B_{12} synthesisor or not. Scott and Ericson (1955) using Co^{60} found no trace of vitamin B_{12} in *Rhodymenia palmata* though they found it in some unidentified organic compound. Another source of vitamins for *E. mutabilis* was suggested by Lieb (1971). The author reported sighting some motile 'bacteria like' particles (0.5 - 1.0 µm) moving

inside the cell. Surek and Melkonian (1981) also reported intracellular bacteria inside *E. mutabilis*. Lieb is of the opinion that these 'bacteria'(seen when fixed in Transeau's preservative made up of one part formaldehyde, three parts 95% ethyl alcohol and six parts water) provided the necessary vitamins in exchange for space in a symbiotic relationship. While the presence of these 'motile bacteria like particles' is confirmed within cells fixed in Transeau's preservative, phase contrast microscopic examination of cells at different phases of growth, sonicated cell contents and electron microscopy did not reveal the presence of any bacteria. Also, sonicated cell contents incubated in different bacterial growth media at different pH did not produce any bacterial growth.

While the ability to utilize organic substrates for energy is vital for organisms including some colourless algae that are devoid of chlorophyll, some photosynthetic algae have the capacity to utilize organic compounds both in light and in darkness (Section 1.7). With *E. mutabilis* it was seen that they could use a number of organic carbon substrates (in the presence of atmospheric CO_2) both in light and in the dark. Glucose brought about more yield compared to other carbon substrates used. With acetate, initial experiments with 0.01M concentration was seen to be toxic and as such the concentration was reduced to 0.001M. In all three strains tested yield was least in acetate. Their inability to utilize 0.01M concentration may be due to the low pH (2.6) as reported by Samejima and Myers (1958) where the authors reported that acetate was toxic to *Chlorella pyrenoidosa* at a concentration of 0.004M at pH 4.5 while the threshold was 0.12M at pH 6.7.

Of the different organic nitrogen substrates tested, there was no growth in the dark in any of the substrates. With photoheterotrophic growth there was measureable growth (yield) when subcultured in asparagine-nitrogen and glycyl-glycine-nitrogen, using inocula from the basal medium in 20 days of incubation. For the other substrates (Table 2.4) repeated subculture in the organic substrate was necessary to induce utilization of organic nitrogen. As *E. mutabilis* utilized organic nitrogen in the light and not in the dark it appears that nitrogenase activity is somehow restricted in the dark. Of the different organic substrates used by *E. mutabilis*, yield was least in urea. The poor yield in urea may be due to insufficient urase produced by the alga, yet urea can be utilized by *Chlorella* in the absence of urase (Syrett, 1962). Thus leading to some other explanation.

Having established that the different organic phosphates are stable over the experimental period of 20 days and that there is both acid and alkaline phosphatase activity in *E. mutabilis D464*, experiments carried out to compare yield during utilization of organic substrates with inorganic P, indicate a 'preference' to orthophosphate as yield was more in PO_4-P compared to the organic substrates used.

4.3 Influence of Heavy Metals

Although all streams have metals in solution, acid streams in particular have a number of toxic heavy metals (Section 1.4). In such acid environments *E. mutabilis* is suggested to be an early coloniser (Sheath *et al.*, 1982). Experiments were designed

and carried out to check the influence of a number of heavy metals (Table 2.5), on yield in other strains of *E. mutabilis* D464,D640,D641 isolated from different areas of known water chemistry (Table 2.7). Of the different heavy metals tested Al had the least toxic effect on yield even with 1000 mg l^{-1} Al in the growth medium. Least tolerance was seen in Cd where 1.0 mg l^{-1} Cd resulted in complete stoppage of growth. Although the concentration of Fe in Brandon Acid Stream from which strain D464 was isolated was 82 mg l^{-1}, yet this strain was not able to tolerate above 10 mg l^{-1} in the basal medium. This was the case with the other two strains. Although some of the concentrations of metals used in the toxicity test are more than the levels met with in natural habitats, it is possible to speculate that with the different metals tested the tolerance will be as follows : Al>Mn>Pb>Ni>Co>Zn>Cu>Fe>Cd. Within the time available it was not possible to check the rate of accumulation of the different metals and the influence of the various permutations of metal tolerance. Instead, as improved yield was seen with increased Zn (10 mg l^{-1}) a number of factors influencing Zn toxicity were tested using strain D464.

4.4 Factors influencing zinc toxicity

The influence of a number of factors influencing Zn toxicity at sublethal (40 mg l^{-1}) and inhibitory (20 mg l^{-1}) level of Zn in the growth medium was examined. It was found that to have a measureable yield in 20 days of incubation a minimum inoculum size was necessary. This inoculum size (10^2 cells ml^{-1}) was used in all the experiments.

Another factor influencing Zn toxicity was the pH of the growth medium. It was found that toxicity was least at pH 3.4

and it increased when the pH was raised above 4.0 with no yield at pH 5.5.

Of the different major cations, sodium and calcium increased toxicity, while magnesium and potassium had no effect on Zn toxicity. It was hypothesised that increased toxicity seen with increased Na was due to the extrusion of K ions due to the Na/K 'pump'. It was found that in the presence of 25 mg l^{-1} Na, toxicity to sublethal Zn levels decreased with increased K, thus suggesting the possible action of the Na/K 'pump' in action. In *Hormidium rivulare* Hargreaves and Whitton (1976) found that in low pH (2.7) the alga grew in the presence of 30 mg l^{-1} Zn in a medium with 100 mg l^{-1} Ca but was able to withstand only 10 mg l^{-1} Zn in a medium with 10 mg l^{-1} Ca. Thus it appears that in *H. rivulare* increased calcium concentration will result in the reduction of zinc toxicity. But in *E. mutabilis* increased zinc toxicity was seen with increased calcium.

Chlorine is said to be the major halogen in most algae, the bulk of which is present as chloride or as organic compound (O'Heocha *et al*, 1958). In *E. mutabilis* with chloride there was a marked reduction in Zn toxicity when the concentration of Cl was increased from 35 mg l^{-1} to 75 mg l^{-1}. With further increase in the Cl concentration above 75 mg l^{-1} there was an increase in Zn toxicity. Thus it appears that with concentrations up to and including 75 mg l^{-1}, Cl has the effect of reducing Zn toxicity whereas with further increases a synergistic effect is seen; this may be due to the binding of some essential nutrients that become unavailable for metabolic activities.

Increased levels of glycyl-glycine-nitrogen reduced Zn toxicity at sublethal Zn level whereas ammonium-nitrogen increased Zn toxicity in *E. mutabilis*. This may be due to Zn being bound to organic substrates and thus becoming unavailable to the organism. Yet with organic phosphate supplied as β-glycerophosphate toxicity to Zn at sublethal Zn level increased.

Of the different heavy metals checked for synergism and antagonism with Zn, it was found that Cu, Fe, Cd, Ni, Co and Al above 100 mg l^{-1} showed increased toxicity at sublethal Zn levels while Mn and Al below 100 mg l^{-1} had no effect on Zn toxicity. Peterson (1968) reported that although Zn is an essential trace element for *Scenedesmus quadricauda* in the presence of Cu it is toxic. A similar synergistic reaction seems to take place in *E. mutabilis*.

Concluding remarks

All four strains of *Euglena mutabilis*, though isolated from different field pH, had optimum yield between pH 3.4 and 4.0. They had a wide pH range for growth and had similar nutritional requirements and tolerance to heavy metals. Strain D464 isolated from a field Zn concentration of 1.1 mg l^{-1} and D641 from 21 mg l^{-1} tolerated the same Zn concentration under laboratory conditions.

Although toxic effect of high concentration of different heavy metals seems to affect the usual reproductive process of binary fission, it was not possible to establish a single factor responsible for tolerance to heavy metals.

E. mutabilis seems to be well adapted to live in low pH

and high concentrations of a number of heavy metals which are toxic to most algae. Lloyd (1977) reported that heavy metals are not available for uptake because of the marked changes brought about by low pH. The author reported that many cations exist in true aqueous solutions at low pH (pH 3.0) and that ions are not as well absorbed as other inorganic complexes. Hargreaves (1977) postulated that hydrogen ions may form a 'barrier' preventing toxic substances from entering the cell. Bachmann (1963) found Zn^{65} uptake by *Golenkinia paucispina* cells was reduced more by hydrogen ions than Ca, Mg, Na and K in that order. Patterson (1983) working on Zn accumulation in *Mougeotia* strain isolated from stream (Durham Code No. 0097) reported that *Mougeotia* accumulated extremely high concentrations of Zn from stream water and hypothesised a 'detoxification process' within the cell as a means of tolerance. Examination of water chemistry data of nine acid streams in England (Appendix 3) indicated that *E. mutabilis* was found in all but two streams (Stream No. 0137 and 0158). The only common denominator to be found is the low Zn level in these two streams.

Either by preventing the entry of toxic heavy metals (Hargreaves 1977), or by a process of detoxification (Patterson, 1983), *E. mutabilis* appears to be well adapted to occupy newly formed acid environments. As no measurements of accumulation of heavy metals was carried out, it is not possible to say whether *E. mutabilis* accumulates heavy metals or not. Yet the presence of an active contractile vacuole tempts to hypothesise an active or a passive intake of, and an active energy

requiring expulsion of, heavy metals by means of the contractile vacuole.

SUMMARY

(a) There are a number of bacteria and fungi associated with the algae in acid environments. Clonal axenic cultures of *Euglena mutabilis* and other unicellular green algae were produced without using any antibiotics, thus avoiding possible risk of mutation.

(b) *E. mutabilis* isolated from different field pH values (1.9, 2.6, 3.5 and 3.7) has a pH optima between pH 3.4 and 4.0. It was possible to adapt strain D464 isolated from, and maintained at pH 2.6, to grow at pH 1.5 and 8.5 by continuous subculture.

(c) Although *E. mutabilis* D 464 grows in the vitamin-free basal medium there was improved yield in the presence of vitamin B_1 and B_{12} with higher yield in vitamin B_1 compared with vitamin B_{12}.

(d) With the macroelements tested, (Na, K, Mg and Ca), there was improved yield in strain D464, D640 and D641 with increased sodium concentration up to and including 25 mg l^{-1}.

(e) Yield improved with Zn, Cu, Mn and Co in microquantities, in *E. mutabilis* D464, D646 and D641. Of the trace elements tested there was improved yield at 10 mg l^{-1} Zn compared to the basal medium with 0.013 mg l^{-1}.

(f) A number of organic substrates were tested both for their suitability for heterotrophic and photoheterotrophic growth. With organic carbon all tests showed a positive effect.

With acetate, however, a lower concentration of (0.001M) was needed as growth was suppressed at 0.01M. There was growth in all organic nitrogen substrates tested in the light and not in the dark. *E. mutabilis* D464 shows both acid and alkaline cell surface phosphatase activity.

(g) The influence of a number of heavy metals was examined and it was found that *E. mutabilis* D464, D640 and D641 is tolerant to a number of heavy metals to varying degrees. Aluminium was the least toxic metal in that yield was not reduced even at a concentration of 1000 mg l^{-1}. Cadmium was the most toxic metal, suppressing growth at 1.0 mg l^{-1}. Tolerance in diminishing order appears to be as follows: Al>Mn>Pb>Ni>Co>Zn>Cu>Fe>Cd.

(h) The effect of a number of environmental factors on zinc toxicity in *E. mutabilis* D464 was checked and it was found that the inoculum size, pH and the composition of the growth medium had a profound influence on Zn toxicity. Toxicity to Zn increased in the presence of Na (10 mg l^{-1}), Ca (10 mg l^{-1}), Cu (0.01 mg l^{-1}), Fe (1.0 mg l^{-1}), Pb (0.1 mg l^{-1}), Cd (0.005 mg l^{-1}), Ni (0.25 mg l^{-1}) and Al (above 100 mg l^{-1}) while it decreased with Cl (up to and including 75 mg l^{-1}) and glycyl-glycine. K (160 mg l^{-1}), Mg (200 mg l^{-1}) and Mn (80 mg l^{-1}) had no effect on Zn toxicity.

APPENDIX ONE

INFLUENCE OF DIFFERENT INORGANIC NITROGEN SUBSTRATES ON GROWTH IN *EUGLENA MUTABILIS*

The effect of different inorganic nitrogen substrates on growth in *Euglena mutabilis* was investigated with inocula from pH 2.6 with strains D464, D640 and D641, in both nitrogen as ammonium and as nitrate in the basal medium. Yield in 20 days at 25°C and photon flux density of 100 μmol m^{-2} s^{-1} (Fig. A.1) indicated utilization of nitrogen only as ammonium and not nitrate. Repeated subculture in nitrate did not induce growth.

Figure A.1 Influence of Nitrate on yield in *Euglena mutabilis*

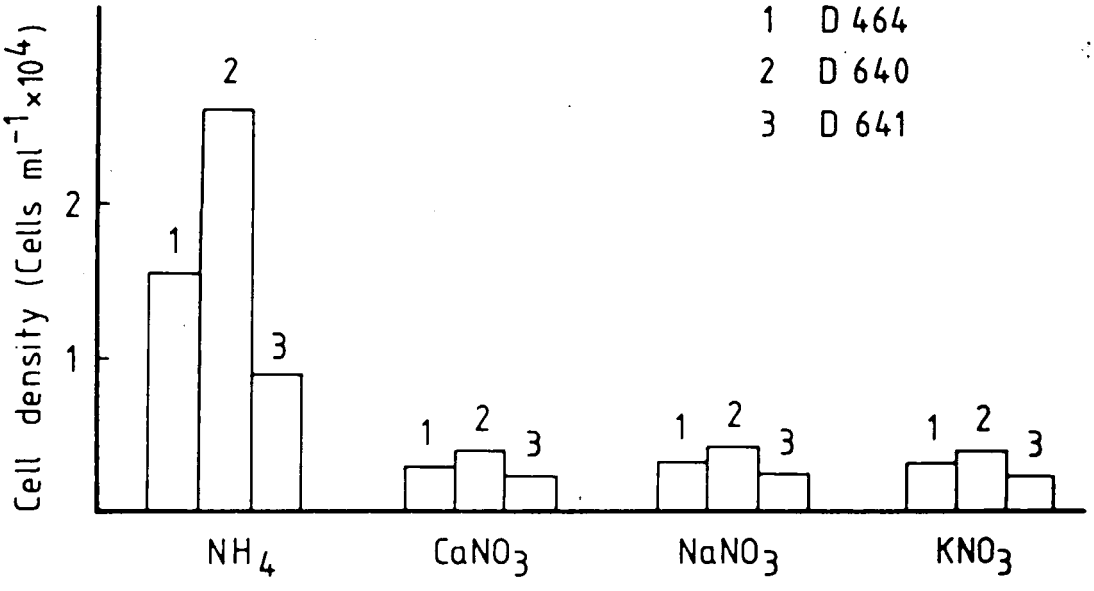

1 D 464
2 D 640
3 D 641
Cell density (Cells ml^{-1} x10^4)
1
2
NH$_4$
CaNO$_3$
NaNO$_3$
KNO$_3$

TABLE A.1 Influence of different inorganic nitrogen substrates on yield (dry weight) in *E. mutabilis* in 20 days at 25°C and photon flux density of 100 µmol m^{-2} s^{-1}. n = 16.

	D464		D640		D641	
Nitrogen substrate	mg ml^{-1}	sd	mg ml^{-1}	sd	mg ml^{-1}	sd
NH_4	0.51	0.01	0.67	0.01	0.31	0.04
$CaNO_3$	0.12	0.01	0.13	0.01	0.12	0.01
$NaNO_3$	0.13	0.02	0.11	0.02	0.13	0.02
KNO_3	0.11	0.02	0.13	0.01	0.11	0.01

APPENDIX TWO

STABILITY OF ORGANIC P IN ACID MEDIUM

Experiments conducted to see if organic P remains stable over the normal incubation period of 20 days (Section 2.477) showed that organic P was stable over the incubation period of 20 days at pH 2.6 at 25^{o}C and photon flux density of 100 µmol m^{-2} s^{-1}; (Table A.2).

TABLE A.2 Fate of organic P in the growth medium

Substrate	day 1 (mg l^{-1})			day 5 (mg l^{-1})			day 10 (mg l^{-1})			day 20 (mg l^{-1})		
	T.P.	S.R.P.	SRP/T.P.	T.P.	S.R.P.	SRP/T.P.	T.P.	S.R.P.	SRP/T.P.	T.P.	S.R.P.	SRP/T.P.
β-Glycerophosphate	1.54	0.3	19.48	1.46	0.30	20.56	1.43	0.31	20.62	1.42	0.32	22.53
DNA	1.13	0.33	29.20	1.11	0.34	30.63	1.19	0.31	28.18	1.10	0.30	27.22
Phytic Acid	1.53	0.32	20.92	1.37	0.27	19.70	1.31	0.31	22.79	1.35	0.31	22.9
Lecithin	1.56	0.28	17.94	1.34	0.21	15.67	1.32	0.30	22.72	1.31	0.32	24.42
Glucose-I-PO_4	1.34	0.30	22.40	1.28	0.32	25.0	1.26	0.34	26.98	1.25	0.31	24.8

APPENDIX THREE

WATER CHEMISTRY OF LOW pH SITES IN ENGLAND

Stream No. and Name		Grid ref.
0136	Cannock opencast acid stream	SJ 990083
0137*	Polesworth Acid Stream	SK 257939
0138	Bridford Acid Stream	SX 816854
0139	Birch Coppice Acid Stream	SP 255001
0140	Kingsbury Acid Stream	SP 233986
0157	Rowley Acid Stream	SD 858331
0158*	Chrisnall Hall Acid Stream 'B'	SD 549125
0159	Chrisnall Hall Acid Stream 'C'	SD 549125
0160	Denby Acid Stream 'B'	SD 392485

* No *Euglena mutabilis* recorded.

APPENDIX THREE - Water Chemistry of low pH sites in England

Stream No.	136	137	138	139	140	157	158	159	160
pH	2.1	2.5	3.0	2.2	2.9	2.5	3.0	2.7	3.0
Acidity	50000	2100	836.0	25333.3	4533.33	10933.7	1900	7900	13300
O_2	8.7	95.67	0.50	91.33	64.33	36.67	101.0	106.0	82.5
Na	23.7	325.7	16.1	877.7	650.33	593.33	51.7	53.7	218.0
K	3.6	6.66	4.65	2.61	7.63	6.35	16.5	16.0	4.85
Mg	278.0	197.7	22.83	651.0	362.0	748.8	505.0	425.0	602.0
Ca	401.0	251.0	49.73	275.33	373.0	441.67	397.0	425.0	317.5
Zn	62.0	0.69	82.67	18.27	34.06	6.25	0.65	1.16	5.95
Cu	3.2	0.05	0.70	2.22	0.24	0.09	0.22	0.81	0.66
Mn	120.0	44.83	29.0	117.97	100.93	162.67	28.9	81.0	81.2
Fe	10000.0	103.31	76.1	3686.67	202.67	3753.33	10.1	1250.0	1825.0
Al	501.0	3.71	8.12	1092.0	298.67	168.33	55.0	130.0	299.0
Pb	0.1	0.15	1.16	1.12	0.25	0.06	0.18	0.34	0.18
Co	4.1	1.62	0.28	6.33	2.17	2.24	0.55	1.21	1.71
Ni	3.1	2.87	1.32	16.2	5.27	4.09	3.05	5.5	6.5
PO_4-P	0.8	0.1	0.14	5.12	0.1	0.19	0.29	0.57	2.0
NH_4-N	4.0	3.19	0.39	2.85	4.59	5.87	1.1	1.41	1.49
SO_4-S	3642.0	950.0	460.3	5189.33	1001.1	4125.0	1014.0	1443.0	1727.0
Cl	32.0	185.0	28.5	123.57	186.67	27.67	29.5	29.0	22.75
Si	11.5	17.67	25.37	46.33	11.25	25.5	26.0	41.0	32.0

APPENDIX FOUR

AC MICROELEMENT STOCK

(low Mn level)

Salt	M.Wt.	quantity in g l^{-1}	using 1 ml l^{-1} element in mg l^{-1}
H_2Bo_3	61.84	2.86	0.5
$MnCl_2 \cdot 4H_2O$	198.	0.181	0.05
$ZnSO_4 \cdot 7H_2O$	287.6	0.222	0.05
$CuSO_4 \cdot 5H_2O$	249.69	0.079	0.02
$CoSO_4 \cdot 7H_2O$	281.13	0.042	0.008
$N_2MoO_4 \cdot 2H_2O$	241.95	0.027	0.010

Made up to one litre with deionised double distilled water and 0.25 ml of AC microelement stock was added to a litre of growth medium.

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